INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	14792094
	Filing Date	2015-07-06
	First Named Inventor	BRENNER, Sydney
	Art Unit	1636
	Examiner Name	BHAT, NARAYAN KAMESHWAR
	Attorney Docket Number	CGLC-020CON5

CERTIFICATION STATEMENT

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

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

OR

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

See attached certification statement.

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

☒ A certification statement is not submitted herewith.

SIGNATURE

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

Signature	/James S. Keddie, Reg. No. 48,920/	Date (YYYY-MM-DD)	2016-06-29
Name/Print	James S. Keddie	Registration Number	48920

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

Privacy Act Statement

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

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

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

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

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

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

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

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

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

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

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

PTO/SB/06 (09-11)
Approved for use through 1/31/2014 OMB 0651-0032
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/792,094	Filing Date 07/06/2015	☐ 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), (l), 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 06/29/2016

	(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))	* 16	Minus	** 20	= 0	X $40 =	0
Independent (37 CFR 1.16(h))	* 3	Minus	***3	= 0	X $210 =	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.

LIE
/STELLA LITTLE/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Electronically Filed

REQUEST TO CHANGE THE APPLICANT UNDER 37 C.F.R. § 1.46(c) Address to: Mail Stop Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450		
	Attorney Docket No.	CGLC-020CON5
	Confirmation No.	9697
	First Named Inventor	Brenner, Sydney
	Application Number	14/792,094
	Filing Date	July 6, 2015
	Group Art Unit	1634
	Examiner Name	BHAT, NARAYAN KAMESHWAR
	Title:	*"Compositions and Methods for Intramolecular Nucleic Acid Rearrangement"*

Dear Sir:

Applicants respectfully request that the above-referenced application be amended to change the Applicant.

Please remove [[**Population Genetics Technologies Ltd.**]] and add **10X Genomics, Inc.** as the sole Applicant. A Supplemental Application Data Sheet in compliance with 37 CFR 1.76 is submitted herewith along with a statement under 37 CFR 3.37(c) to show chain of title to the new applicant.

Applicants also request that a Corrected Filing Receipt be issued for the above-identified application.

If for any reason a fee is found to be necessary, the Commissioner is authorized to charge such fee to Deposit Account No. 50-0815 order number CGLC-020CON5.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: July 13, 2016

By: /James S. Keddie, Reg. No. 48,920/
James S. Keddie
Registration No. 48,920

Bozicevic, Field & Francis LLP
1900 University Avenue, Suite 200
East Palo Alto, California 94303
Telephone: (650) 327-3400
Facsimile: (650) 327-3231

PTO/AIA/96 (08-12)
Approved for use through 01/31/2013. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: **10X Genomics, Inc.**

Application No./Patent No.: **14/792,094** Filed/Issue Date: **July 6, 2015**

Titled: **Compositions and Methods for Intramolecular Nucleic Acid Rearrangement**

10X Genomics, Inc., a **corporation**
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

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

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

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

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

   ☐ There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. ☐ The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. ☐ The recipient, via a court proceeding or the like (*e.g.,* bankruptcy, probate), or an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

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

A. ☐ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.

B. ☒ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: **the inventors** To: **Population Genetics Technologies Ltd**

   The document was recorded in the United States Patent and Trademark Office at

   Reel **036049**, Frame **0472**, or for which a copy thereof is attached.

2. From: **Population Genetics Technologies Ltd** To: **10X Genomics, Inc.**

   The document was recorded in the United States Patent and Trademark Office at

   Reel _____, Frame _____, or for which a copy thereof is attached.

This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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

PTO/AIA/96 (08-12)
Approved for use through 01/31/2013. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

STATEMENT UNDER 37 CFR 3.73(c)

3. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

4. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

5. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

6. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

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

☒ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

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

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

/James S. Keddie, Reg. No. 48,920/	**July 12, 2016**
Signature	Date
James S. Keddie	**48,920**
Printed or Typed Name	Title or Registration Number

INTELLECTUAL PROPERTY ASSIGNMENT AGREEMENT

This **INTELLECTUAL PROPERTY ASSIGNMENT AGREEMENT** ("**IP Assignment**"), dated as of June 7, 2016, is made by Population Genetics Technologies Ltd, a corporation existing under the laws of England and Wales with company number 05116842 and registered office at Salisbury House, Station Road, Cambridge, CB1 2LA, United Kingdom ("**PGT**") and 10X Genomics, Inc., with offices at 7068 Koll Center Pkwy #401, Pleasanton, CA 94566 ("**10X**"), the purchaser of certain assets of PGT pursuant to the Intellectual Property Purchase and License Agreement between PGT and 10X, dated as of October 21, 2015 (the "**IP Purchase Agreement**").

WHEREAS, under the terms of the IP Purchase Agreement, PGT has conveyed, transferred and assigned to 10X, among other assets, certain intellectual property of PGT, and has agreed to execute and deliver this IP Assignment, for recording with governmental authorities including, but not limited to, the US Patent and Trademark Office;

NOW THEREFORE, the parties agree as follows:

1. Assignment. In consideration for the receipt by PGT of the Purchase Price stipulated in the IP Purchase Agreement and other good and valuable consideration, the receipt and sufficiency are hereby acknowledged, PGT hereby irrevocably conveys, transfers and assigns to 10X, and 10X hereby accepts, all of PGT's right, title and interest in and to the patents and patent applications set forth in Schedule 1 hereto and all issuances, divisions, continuations, continuations-in-part, reissues, extensions, reexaminations and renewals thereof (the "**Patents**").

2. Recordation and Further Actions. PGT authorizes the Commissioner for Patents and any other governmental officials to record and register this IP Assignment upon request by 10X. PGT shall, at 10X's cost, take such steps and actions following the date hereof, including the execution of any documents, files, registrations, or other similar items, to ensure that the Assigned IP is properly assigned to 10X, or any assignee or successor thereto.

3. Terms of the IP Purchase Agreement. The terms of the IP Purchase Agreement, including, but not limited to, the representations, warranties, covenants, agreements and indemnities relating to the Assigned IP are incorporated herein by this reference. The parties hereto acknowledge and agree that the representations, warranties, covenants and agreements contained in the IP Purchase Agreement shall not be superseded hereby but shall remain in full force and effect to the full extent provided therein. In the event of any conflict or inconsistency between the terms of the IP Purchase Agreement and the terms hereof, the terms of the IP Purchase Agreement shall govern.

4. Counterparts. This IP Assignment may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this IP Assignment delivered by facsimile, e-mail or other means

of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this IP Assignment.

5. Successors and Assigns. This IP Assignment shall be binding upon and shall inure to the benefit of the parties hereto and their respective successors and assigns.

6. Governing Law. This IP Assignment and any dispute, claim or matter arising from or in connection with it or its subject matter or formation (including non-contractual disputes or claims) shall be governed by and construed in accordance with the law of England and Wales. The parties agree that the courts of England and Wales will have non-exclusive jurisdiction to settle any such dispute or claim and irrevocably agree to submit to the jurisdiction of the courts of England and Wales for these purposes.

[SIGNATURE PAGE FOLLOWS]

IN WITNESS WHEREOF, PGT has duly executed and delivered this Intellectual Property Assignment Agreement as of the date first above written.

POPULATION GENETICS TECHNOLOGIES LTD

By: _____
Name: Mel N. Kronick
Title: Director

Address for Notices:

Population Genetics Technologies Ltd
Salisbury House
Station Road
Cambridge
CB1 2LA
United Kingdom

AGREED TO AND ACCEPTED:

10X GENOMICS, INC.

By: _____
Name: Matthew Murphy
Title: Vice President and General Counsel

Address for Notices:

7068 Koll Center Pkwy #401
Pleasanton
CA 94566
United States of America

SCHEDULE 1

ASSIGNED PATENTS AND PATENT APPLICATIONS

PGT Name	Entity	App No	Date Filed	Pat No	Date Issued	Status
CGLC-020PRV	US	61/235,595	8/20/2009			Expired
CGLC-020PRV2	US	61/288,792	12/21/2009			Expired
CGLC-020WO	WIPO	IB2010/002243	8/13/2010			Expired
CGLC-020EP	EP	10768061.3	8/13/2010	2467479		Registered in DE, ES, FR, GB
CGLC-020EPDIV	EP	15193733.1	8/13/2010			Pending
CGLC-020	US	13/387,343	2/15/2012	8,298,767	10/30/2012	Issued
GCLC020CON	US	13/622,872	9/19/2012			Abandoned
CGLC-020CON2	US	13/859,450	4/09/2013	8,563,274	10/22/2013	Issued
CGLC-020CON3	US	14/021,790	9/09/2013	8,679,756	03/25/2014	Issued
CGLC-020CON4	US	14/172,694	2/04/2014	9,102,980	08/11/2015	Issued
CGLC-020CON5	US	14/792,094	7/06/2015			Pending
CGLC-020CON6	US	14/935,255	11/6/2015			Pending
CGLC-020CON7	US	14/939,728	11/12/2015			Pending

Document Description: Application Data Sheet to update/correct info
Doc Code: ADS.CORR

CORRECTED ADS FORM

Application Number	14792094
Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement

Inventor Information

****If no data is shown, no data has been corrected****

	Data of Record	Updated Data
Order Number		
Name		
Residence Information		
Residency		
City		
State		
Country of Residence		
Mailing Address of Inventor		
Address 1		
Address 2		
City,State/Province, Postal Code		
Country		

Corrected ADS 1.0

Document Description: Application Data Sheet to update/correct info
Doc Code: ADS.CORR

Application Information

	Data of Record	Updated Data
Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement	
Attorney Docket Number	CGLC-020CON5	
Entity Type	Small	

Domestic Benefit/National Stage Information

****If no data is shown, no data has been corrected****

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121,365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S. C. 119(e) or 120, and 37 CFR 1.78(a).

		Data of Record	Updated Data
	Prior Application Status		
	Application Number		
	Continuity Type		
	Prior Application Number		
	Filing Date (YYYY-MM-DD)		
	Patent Number		
	Issue Date (YYYY-MM-DD)		

Foreign Priority Information

Corrected ADS 1.0

Document Description: Application Data Sheet to update/correct info
Doc Code: ADS.CORR

****If no data is shown, no data has been corrected****

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

		Data of Record	Updated Data
	Application Number		
	Country		
	Filing Date		
	Access Code		

Applicant Information

****If no data is shown, no data has been corrected****
Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

	Data of Record	Updated Data
Applicant Type	ASG	
If applicant is the legal representative, indicate the authority to file the patent application, the inventor is		
Name of the Deceased or Legally Incapacitated Inventor		
Applicant is an Organization	Yes	
Name		
Organization Name	~~Population Genetics Technologies LTD.~~	10X Genomics, Inc.
Address 1		7068 Koll Center Pkwy
Address 2		#401

Corrected ADS 1.0

Document Description: Application Data Sheet to update/correct info
Doc Code: ADS.CORR

City,State/Province,Postal Code	~~Cambridge~~	Pleasanton CA 94566
Country	~~GB~~	US
Phone Number		
Fax Number		
Email Address		

Assignee Information including Non-Applicant Assignee Information

****If no data is shown, no data has been corrected****
Providing this information in the application data sheet does not substitute for compliance with any requirement of part 3 of Title 37 of the CFR to have an assignment recorded in the Office

	Data of Record	Updated Data
Order		
Applicant is an Organization		
Name		
Organization Name		

Mailing Address

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

Corrected ADS 1.0

Document Description: Application Data Sheet to update/correct info
Doc Code: ADS.CORR

Signature

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b).

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

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

Signature	/James S. Keddie, Reg. No. 48,920/	Registration Number	48920
First Name	James S.	Last Name	Keddie

Corrected ADS 1.0

Electronic Acknowledgement Receipt	
EFS ID:	26331044
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	
Customer Number:	93726
Filer:	James Samuel Keddie
Filer Authorized By:	
Attorney Docket Number:	CGLC-020CON5
Receipt Date:	13-JUL-2016
Filing Date:	06-JUL-2015
Time Stamp:	20:51:56
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	CGLC-020CON5-Req_Corr_Applcnt.pdf	24756 60d397c6e133aca1fae7fd25623dea9400 3f266	no	1
Warnings:					

Information:					
2	Assignee showing of ownership per 37 CFR 3.73	CGLC-020CON5-373C_Statement.pdf	32443 a600230a6dd805cadaa6c5d140d223e30c038e18	no	2
Warnings:					
Information:					
3	Assignee showing of ownership per 37 CFR 3.73	2016-06-07-PGT-10X-Patent-Assignment-Agreement.pdf	126517 620c91335799a56ce7ce2a0a3565a8d61c09e939	no	4
Warnings:					
Information:					
4	Application Data Sheet to update/ correct info	CorrectedADS.pdf	65647 0c0cd1e75ac4cf3e1e1472631f3cd2ac7f2ac2ec	no	5
Warnings:					
Information:					
		Total Files Size (in bytes):	249363		

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

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

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/792,094	07/06/2015	Sydney Brenner	CGLC-020CON5	9697

93726 7590 07/25/2016

EPA - Bozicevic Field & Francis LLP
Bozicevic, Field & Francis
1900 University Ave
Suite 200
East Palo Alto, CA 94303

EXAMINER
BHAT, NARAYAN KAMESHWAR

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
07/25/2016	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):

docket@bozpat.com

Examiner-Initiated Interview Summary	**Application No.**	**Applicant(s)**	
	14/792,094	BRENNER ET AL.	
	Examiner	**Art Unit**	
	NARAYAN BHAT	1634	

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

(1) *Examiner: Bhat*. (3)_____.

(2) *Representative: Mr. Keddie*. (4)_____.

Date of Interview: *11 July 2016*.

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: *21,35 and 37*.

Identification of prior art discussed: *Arts of the record*.

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...)

See Continuation Sheet.

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of 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

/NARAYAN BHAT/ Primary Examiner, Art Unit 1634	

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00619

Continuation of Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments:

For compact prosecution the examiner contacted the representative Mr. Keddie on July 11, 2016 to discuss further amendments to place the pending claims in better condition for the allowance. The examiner discussed that the claim amendments of June 29, 2016 requiring same multiplex identifier for the sample and the plurality of segments of the nucleic acids still read on the teachings over Braverman and further amendments and terminal disclaimers are needed to place the pending claims in condition for the allowance.

It was agreed that inclusion of an additional step '(b) iii', of "wherein the multiplex identifier associated with a plurality of nucleic acid molecules of the sample is different from a multiplex identifier associated with a plurality of segments from a particular nucleic acid molecule of the sample", fixing the claim language for any minor informalities, 112 second and fourth paragraphs issues and filing the terminal disclaimers over claims of US 8,298,767, 8,563,274, 8,679,756 and 9,102,980 and over claims of 14/935,255 and 14/939,728 copending applications and their acceptance by the office and identifying the support for the amendments in the specification would overcome the rejections on the record and thus allowable.

The representative informed that the amendments require approval from the applicant. On July 13, 2016 the representative requested to issue an office action in view of not hearing a response from the applicant.

The communication between the examiner and the representative is made of record.

Office Action Summary	Application No. 14/792,094	Applicant(s) BRENNER ET AL.	
	Examiner NARAYAN BHAT	Art Unit 1634	AIA (First Inventor to File) Status No

-- 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 6/29/2016.

☐ 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) 21, 24 and 26-39 is/are pending in the application.

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

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) 21,24 and 26-39 is/are rejected.

8)☒ Claim(s) 28 is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

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

Application Papers

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

11)☒ The drawing(s) filed on 7/6/15 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. 7/11/16.

4) ☒ Other: IMS.

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00621

Final Action

1. The present application is being examined under the pre-AIA first to invent provisions. This office action is in response to the papers filed on June 29, 2016. The petition to make the instant application "special" has been granted by the office on December 22, 2015 and thus has been examined on expedited basis.

The instant application is a continuation of 14/172,694, now U.S. Patent 9,102,980, which is a continuation of 14/021,790, now U.S. Patent 8,679,756, which is a continuation of 13/859,450, now U.S. Patent 8,563,274, which is a continuation of 13/622,872, now abandoned, which is a continuation of 13/387,343, now U.S. Patent 8,298,767, which is a national stage entry of PCT/IB2010/002243, claiming priority from provisional application 61288792, filed on 12/21/2009 and from provisional application 61235595, filed on 08/20/2009.

The amendments to independent claims 21, 35 and 37 changing the scope of the previously rejected said claims necessitated the new grounds of rejections as set forth in this office action. Accordingly ***this action has been made final***.

It is noted that this office action has been issued due to time constraint to move the instant application in allotted time and not able to receive the discussed and agreed amendments on time to place the pending claims in better condition for the allowance.

Interview Summary

2. For compact prosecution the examiner contacted the representative Mr. Keddie on July 11, 2016 to discuss further amendments to place the pending claims in better condition for the allowance. The examiner discussed that the claim amendments of June 29, 2016 requiring same multiplex identifier for the sample and the plurality of segments of the nucleic acids still read on the teachings over Braverman and further amendments and terminal disclaimers are needed to place the pending claims in condition for the allowance.

It was agreed that inclusion of an additional step '(b) iii', of "wherein the multiplex identifier associated with a plurality of nucleic acid molecules of the sample is different from a multiplex identifier associated with a plurality of segments from a particular nucleic acid molecule of the sample", fixing the claim language for any minor informalities, 112 second and fourth paragraphs issues and filing the terminal disclaimers over claims of US 8,298,767, 8,563,274, 8,679,756 and 9,102,980 and over claims of 14/935,255 and 14/939,728 copending applications and their acceptance by the office and identifying the support for the amendments in the specification would overcome the rejections on the record and thus allowable.

The representative informed that the amendments require approval from the applicant. On July 13, 2016 the representative requested to issue an office action in view of not hearing a response from the applicant.

The communication between the examiner and the representative is made of record.

Claim Status

3. In the claim listing of June 29, 2016 claims 21, 24 and 26-39 are pending in this application and are under prosecution. Claims 21, 24, 28, 29, 31 and 35-37 are amended. Claims 1-20, 22 and 23 are canceled. No new matter has been introduced by the claim amendments as applicant has identified the support for the amendments in the instant specification (Remarks, pg. 7).

Withdrawn Objection and Rejections and Response to the Remarks

4. The previous objection to the abstract has been withdrawn in view of amending the abstract in line with the claims (Remarks, pg. 7). The amendments do not introduce the new matter.

The previous objection to the specification not listing the header "Brief Description of the Drawings" was an oversight on the part of the examiner. It is noted that said header is listed in page 1 of the instant specification.

5. The previous ODP rejections of instant claims 21-39 over claims of 7,544,473, 8,685,678, 8,722,368 and over claims of 14/273,197 copending application in view of Braverman has been withdrawn in view of the claim amendments. The arguments regarding said ODP rejections have been fully considered (Remarks, pgs. 7 and 8). They are moot in view of withdrawn rejections necessitated by the claim amendments.

6. The previous rejection of claims 21-25 and 28-39 under pre-AIA 102(b) as being anticipated by Brenner has been withdrawn necessitated by the claim amendments.

The arguments regarding said rejection have been fully considered (Remarks, pg. 8). They are moot in view of withdrawn rejections necessitated by the claim amendments.

Maintained Rejections and Response to the Remarks

7. The previous rejection of claims 21-25 and 28-39 under pre-AIA 35 USC 102(b) as being anticipated by Braverman has been maintained. The arguments regarding said rejection have been fully considered (Remarks, pg. 8). They are not persuasive because claims as amended only require single multiplex identifier that identifies the sample and the segments of nucleic acids recited in generality without distinguishing the segment of the nucleic acid molecule and the segments of the particular nucleic acid molecule. This feature is taught by Braverman.

8. The previous rejection of claims 21, 24 and 26-39 under pre AIA 35 USC 103(a) as being unpatentable over Braverman in view of Bosnes has been maintained. The arguments regarding the said rejection have been fully considered (Remarks, pg. 9). They are not persuasive for the following reasons.

The arguments are directed to Braverman is deficient because it fails to teach or suggest a method for tagging nucleic acid molecules in a way that results in the segments of a particular nucleic acid molecule being tagged with the same tag sequence and Bosnes, which is cited solely to provide a way to analyze mRNA, fails to meet Braverman's deficiency and, as such, the cited references, taken in any combination, fail to provide each and every element of the rejected claims (Remarks, pg. 9).

The above arguments are not persuasive because claims 21, 35 and 37 as amended requiring the multiplex identifier (MID) sequence associated with a segment of a nucleic acid molecule of (a) uniquely identifies a sample and all of the plurality of tagged segments of a particular nucleic acid molecule of (a) are associated with the same MID sequence has been interpreted as to require only one type of MID because the MID sequence associated with a segment of a nucleic acid molecule identifying the sample has been interpreted as to be present in all of the plurality of tagged segments because the claims as recited do not recite any distinguishing features of the nucleic acid molecule of step '(b)i.' and step '(b)ii.'.

Braverman teaches the claimed steps and is not deficient as allegedly asserted by the applicants. Furthermore as discussed in the interview summary, said claims have been further amended to make the pending claims non-obvious improvements over the arts of record. Therefore rejection over Braverman in view of Bosnes has been maintained.

Claim interpretation

9. Instant independent claims 21, 35 and 37 as amended requiring the multiplex identifier (MID) sequence associated with a segment of a nucleic acid molecule of (a) uniquely identifies a sample and all of the plurality of tagged segments of a particular nucleic acid molecule of (a) are associated with the same MID sequence has been interpreted as to require only one type of MID because the MID sequence associated with a segment of a nucleic acid molecule identifying the sample has been interpreted

as to be present in all of the plurality of tagged segments because the claims as recited do not recite any distinguishing features of the nucleic acid molecule of step '(b)i.' and step '(b)ii.'.

The following Objection and rejections are necessitated by the claim amendments.

Double Patenting

10. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the claims at issue are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); In re Vogel, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l) (1) - 706.02(l) (3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

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

11. Claims 21, 24 and 26-39 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-12 of U.S. Patent No. 8,168,385 in view of Braverman et al (US 2009/0105959, cited in the previous office action) and Bosnes (WO 03/062462, cited in the previous office action) for the following reasons.

Regarding instant independent claims 21, 35 and 37, claim 1 of '385 patent is drawn to a method of sample analysis, comprising: a) separately ligating an oligonucleotide tag to each of a plurality of samples that comprise fragmented genomic DNA to produce a plurality of adaptor-ligated samples, wherein: i.) each of said samples is made from a different source; ii.) each of said samples is ligated to a different oligonucleotide tag; iii.) each oligonucleotide tag comprises at least one word comprising a unique nucleotide sequence that distinguishes said oligonucleotide tag from other oligonucleotide tags; and iv.) each adaptor-ligated sample comprises adaptor-ligated genomic fragments that contain an adaptor and a genomic fragment; b) pooling said plurality of adaptor-ligated samples to make a pooled sample; c) isolating from said pooled sample adaptor-ligated genomic fragments that correspond to one or more genomic loci, thereby producing isolated fragments; and d) sequencing an isolated fragment of (c) to obtain: i.) the unique nucleotide sequence of an oligonucleotide tag; and ii.) the nucleotide sequence of at least part of a genomic fragment; wherein the unique nucleotide sequence of said isolated fragment identifies from which of said plurality of different polynucleotide samples said isolated fragment is derived.

One having ordinary skill in the art would recognize that the oligonucleotide tag specific to each sample meets the limitation of the multiplex identifier (MID) sequence associated with a segment of a nucleic acid molecule of (a) uniquely identifies a sample and all of the plurality of tagged segments of a particular nucleic acid molecule of (a) are associated with the same MID sequence. Claims of '385 patent is not drawn to the nucleic acid samples are from a single isolated cell, which is taught by Braverman (Figs. 1 and 2 and paragraphs 0077-0084) for enriching the concentration of nucleic acids from the single cell and to determine the error rate in the sequencing run (paragraph 0082).

With regard to the limitations of dependent claims 24, 26-34, 36, 38 and 39 claims 1-12 of '385 patent in view of Braverman and Bosnes are drawn to the limitations of said dependent claims and therefore are not patentably significant.

12. Claims 21, 24 and 26-39 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-13 of U.S. Patent No. 8,318,433 in view of Braverman et al (US 2009/0105959, cited in the previous office action) and Bosnes (WO 03/062462, cited in the previous office action) for the following reasons.

Regarding instant independent claims 21, 35 and 37 claim 1 of '433 patent is drawn to method of sequencing comprising: sequencing a plurality of genomic fragments, wherein the genomic fragments are obtained from a plurality of different sources, and wherein: i.) each of said genomic fragments comprises an oligonucleotide tag that comprises a unique nucleotide sequence that indicates from which source a

genomic fragment is derived; and, separate from said unique nucleotide sequence, a binding site for a sequencing primer; ii.) said sequencing of the plurality of genomic fragments is done using said sequencing primer; and iii.) said genomic fragments correspond to one or more selected portions of a genome that have been isolated from a pooled genomic sample by specific hybridization; to obtain, for each of the sequenced fragments, the unique nucleotide sequence of an oligonucleotide tag and the nucleotide sequence of at least a portion of a genomic fragment.

Claims of '433 patent is not drawn to the nucleic acid samples are from a single isolated cell, which is taught by Braverman (Figs. 1 and 2 and paragraphs 0077-0084) for enriching the concentration of nucleic acids from the single cell and to determine the error rate in the sequencing run (paragraph 0082).

With regard to the limitations of dependent claims 24, 26-34, 36, 38 and 39 claims 1-13 of '433 patent in view of Braverman and Bosnes are drawn to the limitations of said dependent claims and therefore are not patentably significant.

In response to this office action and depending on the claim amendments, the ODP issue will be revisited at the time of the allowance.

Claim Objections

13. Claim 28 is objected to because of it is dependent from canceled claim 23. For compact prosecution, claim 28 presumed to be dependent from claim 21. If applicant disagree with claim 28 dependency, applicant is suggested to correct its dependency. Appropriate correction is required.

Claim Rejections - 35 USC § 112

14. 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.

15. Claims 21, 24 and 26-39 are 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.

16. Claim 21 recites the limitation "the nucleic acid molecules" in step (b) line 1. There is insufficient antecedent basis for "the" nucleic acid molecules limitation in the claim. Applicant is suggested to provide proper antecedent basis for "the" nucleic acid molecules limitation in the claim.

17. Claims 24 and 26-34 are indefinite because they are dependent from claim 21.

18. Claim 24 recites the limitation "the product" in line 1. There is insufficient antecedent basis for "the" product limitation in the claim. Applicant is suggested to provide proper antecedent basis for "the" product limitation in the claim

19. Claim 35 recites the limitation "the nucleic acid molecules" in line 4. There is insufficient antecedent basis for "the" nucleic acid molecules limitation in the claim. Applicant is suggested to provide proper antecedent basis for "the" nucleic acid molecules limitation in the claim.

20. Claim 36 is indefinite because it is dependent from claim 35.

21. Claim 37 recites the limitation "the nucleic acid molecules" in line 4. There is insufficient antecedent basis for "the" nucleic acid molecules limitation in the claim. Applicant is suggested to provide proper antecedent basis for "the" nucleic acid molecules limitation in the claim.

22. Claims 38 and 39 are indefinite because they are dependent from claim 37.

For compact prosecution, applicant is suggested to carefully review the claims for any minor informalities and 112 second and fourth paragraph issues and further clarify the steps of distinguishing the multiplex identifier (MID) sequence associated with a segment of a nucleic acid molecule of (a) uniquely identifies a sample and all of the plurality of tagged segments of a particular nucleic acid molecule of (a) are associated with the same MID sequence because of lack of distinguishing features of "a nucleic acid molecule" of step '(b)i.' from "a particular nucleic acid molecule" from step '(b)ii.'.

Claim Rejections - 35 USC § 102

23. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

The following rejection is based on the interpretation of claims 21, 35 and 37 requiring only one multiplex identifier based on the interpretation of amended claim as discussed in section 9.

24. Claims 21, 24 and 28-39 are rejected under pre-AIA 35 U.S.C. 102(a) and 102(e) as being anticipated by Braverman et al (US 2009/0105959, cited in the previous office action).

Regarding claims 21 and 25, Braverman method of analyzing nucleic acids, comprising providing a plurality of nucleic acids from a single cell source (paragraph 0077) and tagging (i.e., associating) the plurality of nucleic acids from the single cell source with an unique identifier (UID) element also referred as multiplex identifier (MID; paragraph 0003) and determining a nucleotide sequence of the plurality of nucleic acids and attached MID sequences, wherein the MID sequences uniquely identify the single cell source, wherein the MID sequences uniquely identify each of a plurality of nucleic acid molecules from the single cell source (Fig. 2 and paragraphs 0003, 0077, 0082-0088). As discussed in the claim interpretations section, since there are no distinguishing features between "a nucleic acid molecule" of 'step (b)i' and 'a particular nucleic acid molecule of 'step (b)ii' is encompassed by the MID identifier of the sample.

Regarding claim 24, Braverman teaches an amplification of plurality of nucleic acid attached to the UID sequence before determining the nucleic acid sequence (paragraph 0088).

Regarding claim 28, Braverman teaches identifying a number of the plurality of nucleic acids tagged with a unique MID, and determining a number of the plurality of nucleic acid molecules that were within a single cell source from a number of unique MID sequences identified (Fig. 1 and paragraphs 78-83).

Regarding claim 29, Braverman teaches that the tagging step comprises ligating a sequence comprising the MID sequences to the plurality nucleic acids (paragraphs 0097).

Regarding claim 30, Braverman teaches that the tagging step comprises performing PCR of the plurality of nucleic acids using an adapter sequence comprising the MID sequence and an amplification primer (Fig. 2 and paragraphs 0088 and 0096-0099).

Regarding claims 31 and 32, Braverman teaches providing a plurality of nucleic acids from each of a plurality of single cell sources; the attaching step comprises attaching different MID sequences to the nucleic acids from each of the plurality of single cell sources; and wherein nucleic acid sequences are attributed to different individual cell sources based upon the MID sequences attached in the attaching step and a sequencing primer sequence (Fig. 2 and paragraphs 0085-0088).

Regarding claims 33 and 34, Braverman teaches that the MID sequences are 10-mer in length, which is within the range of 2 to 100 bases in length and at least 144 different MID sequences (paragraph 0092).

Regarding claim 35-37, Braverman teaches a method of analyzing nucleic acids, comprising: providing a plurality of nucleic acids from a single cell source (paragraph 0077). Braverman also teaches tagging (i.e., associating) the plurality of nucleic acids from the single cell source with a unique identifier (UID) element also referred as multiplex identifier (MID; paragraph 0003). Braverman further teaches an amplification of plurality of nucleic acid attached to the UID sequence before determining the nucleic acid sequence (paragraph 0088) and determining a nucleotide sequence of the plurality of nucleic acids and attached MID sequences, wherein the MID sequences uniquely identify the single cell source, wherein the MID sequences uniquely identify each of a

plurality of nucleic acid molecules from the single cell source (Fig. 2 and paragraphs 0003, 0077, 0082-0088). As discussed in the claim interpretations section, since there are no distinguishing features between "a nucleic acid molecule" of 'step (b)i' and 'a particular nucleic acid molecule of 'step (b)ii' is encompassed by the MID identifier of the sample.

Regarding claim 38, Braverman teaches that the amplifying step comprises performing a polymerase chain reaction amplification (paragraph 0086).

Regarding claim 39, Braverman teaches that the amplifying step comprises a linear amplification (i.e., isothermal amplification) step (paragraph 0007).

Claim Rejections - 35 USC § 103

25. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

26. Claims 21, 24 and 26-39 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Braverman et al (US 2009/0105959, cited in the previous office action) in view of Bosnes (WO 03/062462, cited in the previous office action).

Braverman and Bosnes teach a method for analyzing nucleic acid from a sample and therefore are analogous arts. The teachings of Bosnes are specifically applied for the limitations of claims 26 and 27 of nucleic acid is DNA and mRNA for correlating information relating to mRNA to genomic information.

Regarding claims 21 and 25, Braverman method of analyzing nucleic acids, comprising providing a plurality of nucleic acids from a single cell source (paragraph

0077) and tagging (i.e., associating) the plurality of nucleic acids from the single cell source with an unique identifier (UID) element also referred as multiplex identifier (MID; paragraph 0003) and determining a nucleotide sequence of the plurality of nucleic acids and attached MID sequences, wherein the MID sequences uniquely identify the single cell source, wherein the MID sequences uniquely identify each of a plurality of nucleic acid molecules from the single cell source (Fig. 2 and paragraphs 0003, 0077, 0082-0088). As discussed in the claim interpretations section, since there are no distinguishing features between "a nucleic acid molecule" of 'step (b)i' and 'a particular nucleic acid molecule of 'step (b)ii' is encompassed by the MID identifier of the sample.

Regarding claim 24, Braverman teaches an amplification of plurality of nucleic acid attached to the UID sequence before determining the nucleic acid sequence (paragraph 0088).

Regarding claims 26 and 27, Braverman teaches nucleic acids (0018). However, Braverman does not specifically teach that the nucleic acid is DNA and mRNA.

Regarding claim 28, Braverman teaches identifying a number of the plurality of nucleic acids tagged with a unique MID, and determining a number of the plurality of nucleic acid molecules that were within a single cell source from a number of unique MID sequences identified (Fig. 1 and paragraphs 78-83).

Regarding claim 29, Braverman teaches that the tagging step comprises ligating a sequence comprising the MID sequences to the plurality nucleic acids (paragraphs 0097).

Regarding claim 30, Braverman teaches that the tagging step comprises performing PCR of the plurality of nucleic acids using an adapter sequence comprising the MID sequence and an amplification primer (Fig. 2 and paragraphs 0088 and 0096-0099).

Regarding claims 31 and 32, Braverman teaches providing a plurality of nucleic acids from each of a plurality of single cell sources; the attaching step comprises attaching different MID sequences to the nucleic acids from each of the plurality of single cell sources; and wherein nucleic acid sequences are attributed to different individual cell sources based upon the MID sequences attached in the attaching step and a sequencing primer sequence (Fig. 2 and paragraphs 0085-0088).

Regarding claims 33 and 34, Braverman teaches that the MID sequences are 10-mer in length, which is within the range of 2 to 100 bases in length and at least 144 different MID sequences (paragraph 0092).

Regarding claim 35-37, Braverman teaches a method of analyzing nucleic acids, comprising: providing a plurality of nucleic acids from a single cell source (paragraph 0077). Braverman also teaches tagging (i.e., associating) the plurality of nucleic acids from the single cell source with a unique identifier (UID) element also referred as multiplex identifier (MID; paragraph 0003). Braverman further teaches an amplification of plurality of nucleic acid attached to the UID sequence before determining the nucleic acid sequence (paragraph 0088) and determining a nucleotide sequence of the plurality of nucleic acids and attached MID sequences, wherein the MID sequences uniquely identify the single cell source, wherein the MID sequences uniquely identify each of a

plurality of nucleic acid molecules from the single cell source (Fig. 2 and paragraphs 0003, 0077, 0082-0088). As discussed in the claim interpretations section, since there are no distinguishing features between "a nucleic acid molecule" of 'step (b)i' and 'a particular nucleic acid molecule of 'step (b)ii' is encompassed by the MID identifier of the sample.

Regarding claim 38, Braverman teaches that the amplifying step comprises performing a polymerase chain reaction amplification (paragraph 0086).

Regarding claim 39, Braverman teaches that the amplifying step comprises a linear amplification (i.e., isothermal amplification) step (paragraph 0007).

As discussed above regarding claims 26 and 27, Braverman does not specifically teach that the nucleic acid is DNA and mRNA. However, the DNA and mRNA from the same sample were known in the art at the time the claimed invention was made as taught by Bosnes, who teaches a method for isolating DNA and mRNA from the same sample and further teaches having both DNA and mRNA from the same sample allows for correlating information relating to mRNA to genomic information (Abstract).

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the DNA and mRNA from the single cell source of Bosnes in the method of Braverman with a reasonable expectation of success with the expected benefit of correlating information relating to mRNA to genomic information thus understanding the interrelationship of genotype and phenotype as taught by Bosnes (Abstract). An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including the mRNA from

the single cell source because isolation of DNA, mRNA and protein are is routinely practiced in the art as exemplified by Bosnes.

It is further noted that in KSR, the Supreme Court particularly emphasized "the need for caution in granting a patent based on the combination of elements found in the prior art," (USPQ2d at 1395), and reaffirmed principles based on its precedent that "[t]he combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results."

In the instant case, the steps of claims 21, 24 and 26-39 are a combination of known steps which are well understood, routine, conventional steps already engaged in by the scientific community for improving the sensitivity, specificity of the nucleic acid analysis and were known in the art at the time the claimed invention was made. As discussed above, Braverman in view of Bosnes teaches the claimed steps and combined as suggested above would arrive at the claimed invention. Also, one having ordinary skill in the art would recognize that choosing both DNA and mRNA template would be a mere design choice because steps of analyzing mRNA templates would not be different as the steps for converting mRNA to cDNA are routine, conventional and well established in the art. Thus the steps of pending claims when viewed as whole, add nothing significant beyond the sum of the steps taken from Braverman and Bosnes. Therefore the steps of claims 21, 24 and 26-39 do not amount to "significantly more" and thus obvious improvement over Braverman.

27. Claims 21, 24 and 26-39 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Brenner (US 2006/0177832 published Aug. 10, 2006) in view of Braverman et al (US 2009/0105959, cited in the previous office action) and further in view of Bosnes (WO 03/062462, cited in the previous office action).

Brenner, Braverman and Bosnes teach a method for analyzing nucleic acid from a sample and therefore are analogous arts. The teachings of Braverman are specifically applied for the nucleic acid sample from a single cell and the teachings of Bosnes are specifically applied for the limitations of claims 26 and 27 of nucleic acid is DNA and mRNA for correlating information relating to mRNA to genomic information.

Regarding claims 21, 24 and 26-39, Brenner teaches a method comprising providing fragments from a population of genomes, wherein the fragments from each genome has the same unique oligonucleotide tag attached. In this aspect, the invention includes a method of determining a frequency of a nucleotide at a predetermined locus in a population of genomes, such method comprising the following steps: (i) separately generating fragments of each genome of the population; (ii) attaching a unique oligonucleotide tag to each genome; (iii) selecting fragments from each genome that contains the predetermined locus; (iv) generating a labeled oligonucleotide tag from each unique oligonucleotide tag, the labeled oligonucleotide tag generating a signal indicative of the nucleotide at the predetermined locus; and (v) determining the frequency of the nucleotide at the predetermined locus by detecting the signals generated by the labeled oligonucleotide tags specifically hybridized with their respective tag complements, the respective tag complements being attached in spatially

discrete regions on the one or more solid phase supports (paragraph 0008). Brenner also teaches the amplification and sequencing of the nucleic acid fragments (paragraphs 0031 and 0050). As discussed in the claim interpretations section, since there are no distinguishing features between "a nucleic acid molecule" of 'step (b)i' and 'a particular nucleic acid molecule of 'step (b)ii' is encompassed by the MID identifier of the sample.

Brenner does not specifically teach that the nucleic acids are from a single cell, and comprise mRNA. Braverman teaches a method of analyzing nucleic acids, providing a plurality of nucleic acids from a single cell source (paragraph 0077).

Brenner in view of Braverman does not specifically teach that the nucleic acid is DNA and mRNA. However, the DNA and mRNA from the same sample were known in the art at the time the claimed invention was made as taught by Bosnes, who teaches a method for isolating DNA and mRNA from the same sample and further teaches having both DNA and mRNA from the same sample allows for correlating information relating to mRNA to genomic information (Abstract).

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the DNA and mRNA from the single cell source of Bosnes in the method of Brenner in view of Braverman with a reasonable expectation of success with the expected benefit of correlating information relating to mRNA to genomic information thus understanding the interrelationship of genotype and phenotype as taught by Bosnes (Abstract). An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including the

mRNA from the single cell source because isolation of DNA, mRNA and protein are is routinely practiced in the art as exemplified by Bosnes.

Conclusion

28. No claims are allowed.

29. 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 NARAYAN BHAT whose telephone number is (571)272-5540. The examiner can normally be reached on 8.30 am to 7 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave Nguyen can be reached on (571)-272-0731. 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.

/NARAYAN BHAT/
Primary Examiner, Art Unit 1634

Examiner-Initiated Interview Summary	**Application No.**	**Applicant(s)**	
	14/792,094	BRENNER ET AL.	
	Examiner	**Art Unit**	
	NARAYAN BHAT	1634	

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

(1) *Examiner: Bhat*. (3)_____.

(2) *Representative: Mr. Keddie*. (4)_____.

Date of Interview: *11 July 2016*.

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: *21,35 and 37*.

Identification of prior art discussed: *Arts of the record*.

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...)

See Continuation Sheet.

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of 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

/NARAYAN BHAT/ Primary Examiner, Art Unit 1634	

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Continuation of Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments:

For compact prosecution the examiner contacted the representative Mr. Keddie on July 11, 2016 to discuss further amendments to place the pending claims in better condition for the allowance. The examiner discussed that the claim amendments of June 29, 2016 requiring same multiplex identifier for the sample and the plurality of segments of the nucleic acids still read on the teachings over Braverman and further amendments and terminal disclaimers are needed to place the pending claims in condition for the allowance.

It was agreed that inclusion of an additional step '(b) iii', of "wherein the multiplex identifier associated with a plurality of nucleic acid molecules of the sample is different from a multiplex identifier associated with a plurality of segments from a particular nucleic acid molecule of the sample", fixing the claim language for any minor informalities, 112 second and fourth paragraphs issues and filing the terminal disclaimers over claims of US 8,298,767, 8,563,274, 8,679,756 and 9,102,980 and over claims of 14/935,255 and 14/939,728 copending applications and their acceptance by the office and identifying the support for the amendments in the specification would overcome the rejections on the record and thus allowable.

The representative informed that the amendments require approval from the applicant. On July 13, 2016 the representative requested to issue an office action in view of not hearing a response from the applicant.

The communication between the examiner and the representative is made of record.

Notice of References Cited	Application/Control No. 14/792,094	Applicant(s)/Patent Under Reexamination BRENNER ET AL.	
	Examiner NARAYAN BHAT	Art Unit 1634	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-2006/0177832 A1	08-2006	Brenner; Sydney	C12P19/34	435/6.12
*	B	US-8,168,385 B2	05-2012	Brenner; Sydney	C12N15/1065	435/6.11
*	C	US-8,318,433 B2	11-2012	Brenner; Sydney	C12N15/1065	435/6.1
*	D	US-8,298,767 B2	10-2012	Brenner; Sydney	C12Q1/6855	435/287.2
*	E	US-8,563,274 B2	10-2013	Brenner; Sydney	C12Q1/6855	435/287.2
*	F	US-8,679,756 B1	03-2014	Brenner; Sydney	C12Q1/6855	435/287.2
*	G	US-9,102,980 B2	08-2015	Brenner; Sydney	C12Q1/6855	1/1
	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	
	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.

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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	28875	brenner.in. or mikawa.in. or osborne.in. or slatter.in. or ("POPULATION GENETICS TECHNOLOGIES").as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/07/16 18:09
L2	79	1 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier or tag or uniqu$3 or "bar-code" or "bar code")same (nucleic or dna or polynucleotide).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/07/16 18:11
L3	51	2 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier or tag or uniqu$3 or "bar-code" or "bar code")same sample.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/07/16 18:12
L4	18	3 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier or tag or uniqu$3 or "bar-code" or "bar code")same sequencing.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/07/16 18:13
L5	11	4 and ("nucleotide sequence") same ("multiplex identifier" or mid or barcode or zipcode or token or identifier or tag or uniqu$3 or "bar-code" or "bar code").clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/07/16 18:14
S1	28662	brenner.in. or mikawa.in. or osborne.in. or slatter.in. or population.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 15:41
S2	232	S1 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 15:45
S3	12	S2 and ("multiplex identifier" or mid or	US-PGPUB;	OR	ON	2016/01/05

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		barcode or zipcode or token or identifier) same (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) same (nucleic or dna).clm.	USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			15:47
S4	0	S3 and (single or one) near3 (source or cell) same (nucleic or dna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 15:53
S5	10	S3 and (source or cell) same (nucleic or dna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 15:53
S6	65	S1 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) same (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) same (nucleic or dna)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:00
S7	55	S6 and (single or one) same(source or cell) same (nucleic or dna)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:01
S8	8	S1 and "reflex sequence".clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:08
S9	174	("multiplex identifier" or mid or barcode or zipcode or token or identifier) same (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) same (nucleic or dna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:11
S10	25	S9 and (single or one) same (source or cell) same (nucleic or dna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:11
S11	69	("20040224331" | "20060008799" | "20070020640" | "20070236113" | "20070259357" | "20090155780" | "20100086914" | "20100113296" | "20100120098" | "20110287435" | "20120208724" | "20120316074" |	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT;	OR	ON	2016/01/05 16:30

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		"20130059310" | "6723513" | "7544473" | "7700325" | "8101346" | "8298767" | "8318460").PN.	IBM_TDB			
S12	8	S11 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) near5 (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) near5 (nucleic or dna or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:31
S13	138	"7393665"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:36
S14	2	"7393665".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:36
S15	599	("multiplex identifier" or mid or barcode or zipcode or token or identifier) near5 (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) near5 (nucleic or dna or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:37
S16	147	S15 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) near5 (determin$3 or obtain$3) near5 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:38
S17	697052	435/$.ccls. or 506/$.ccls. or 422/$.ccls. or 536/$.ccls. or (c12q1/68 or c07h21/02 or c07h21/04 or c12p19/34 or c12m1/34 or g01n33/52 or c40b30/04 or c40b40/04 or c40b50/04 or g01n31/22).cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:50
S18	481	S17 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) near5 (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) near5 (nucleic or dna or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:51
S19	131	S18 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) near5 (determin$3 or obtain$3) near5 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:51
S20	45	S19 and (single or one) near5 (source or cell) near5 (nucleic or dna or	US-PGPUB; USPAT;	OR	ON	2016/01/05 16:52

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		polynucleotide)	USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			
S21	17	S1 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) same (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) same (nucleic or dna or polynucleotide).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:55
S22	3	S21 and (single or one or indivudual) same (source or cell) same (nucleic or dna or polynucleotide).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:56
S23	3	S21 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) same (determin$3 or obtain$3) same sequence.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:58
S24	101206	("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier").clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:01
S25	101649	("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:01
S26	196	S25 and (dna and rna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:02
S27	72	S26 and (dna and rna) near5 cell	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:03
S28	29	S27 and ("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid)near5 primer	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:04

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S29	23047	extract$3 near5 (dna or nucleic) near5 cell	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:06
S30	1272	S29 and extract$3 near5 (ribonucleic or mrna)near5 cell	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:07
S31	6	S30 and ("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid)near5 primer	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:07
S32	199	S30 and ("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:08
S33	1	S30 and ("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid) near5 track$3 near5 (sample or source)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:09
S34	11	S30 and ("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid) same (track$3 or origin or identif$3) same (sample or source)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:10
S35	31772	("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid) same (track$3 or origin or identif$3) same (sample or source)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:11
S36	505	S35 and amplif$3 same (dna or nucleic) same ("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:12
S37	91	S36 and amplif$3 same (cdna or mrna) same ("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO;	OR	ON	2016/01/06 18:12

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			DERWENT; IBM_TDB			
S38	5559	("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid) near5 (track$3 or origin or identif$3) near5 (sample or source)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:22
S39	41	S38 and extract$3 near5 (ribonucleic or mrna)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:22
S40	70439	genotype and phenotype	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:26
S41	1534	S40 and phenotype near5 mrna	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:27
S42	75	S41 and extract$3 near5 (ribonucleic or mrna)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:27
S43	0	S42 and ("multiplex identifier" or barcode or zipcode or token or "unique identifier")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:28
S44	2218	S40 and ("multiplex identifier" or barcode or zipcode or token or "unique identifier")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:28
S45	1788	S44 and (ribonucleic or mrna)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:29
S46	63	S45 and ("multiplex identifier" or barcode or zipcode or token or "unique identifier") same mrna	US-PGPUB; USPAT; USOCR;	OR	ON	2016/01/06 18:29

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00652

			FPRS; EPO; JPO; DERWENT; IBM_TDB			
S47	153	S40 and ("multiplex identifier" or barcode or zipcode or token or "unique identifier").clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:31
S48	10	S47 and (ribonucleic or mrna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:31
S49	60223	("multiplex identifier" or barcode or zipcode or token or "unique identifier").clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:33
S50	75	S49 and (ribonucleic or mrna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:33
S51	2	(bhat.xa. or bhat.xp.) and (simultan$4 near5 isolat$4)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/07 10:54
S52	103	(bhat.xa. or bhat.xp.) and isolat$4.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/07 10:54

7/16/2016 6:34:16 PM
C:\Users\nbhat1\Documents\EAST\Workspaces\14792094-1-6-16.wsp

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00653

From: James S. Keddie
To: Bhat, Narayan
Subject: Re: 14/792,094 -status update
Date: Wednesday, July 13, 2016 1:46:44 PM

Nope. Please go ahead and send an office action. Thx. James

On Jul 14, 2016, at 1:25 AM, Bhat, Narayan <Narayan.Bhat@USPTO.GOV> wrote:

> Mr. Keddie,
> Good morning. Did you get a response from your client yet? For reducing the number of actions per disposal, I can wait for two more days. Any update from your end is greatly appreciated. You have a good day.
>
> Narayan K Bhat
> Primary Examiner, AU 1634.
> Remsen 3C09, TC 1600, USPTO, DOC, Alexandria, VA-22313
> T: 571-272-5540
> Fax: 571-273-5540
>
> **From:** James S. Keddie [mailto:keddie@bozpat.com]
> **Sent:** Monday, July 11, 2016 4:50 PM
> **To:** Bhat, Narayan <Narayan.Bhat@USPTO.GOV>
> **Subject:** 14/792,094
>
> Dear Exr. Bhat,
>
> I forwarded the proposed amendment to the client earlier today but I haven't heard back. If you don't hear from me in time, please just send out an office action.
>
> Thanks,
>
> James
>
> James Keddie Ph.D.
> Senior Patent Agent
> BOZICEVIC, FIELD & FRANCIS LLP
> 1900 University Ave Suite 200
> East Palo Alto, Ca 94303
> Main: (650) 327-3400; Fax: (650) 327-3231
> Direct: (650) 833-7723; Email: keddie@bozpat.com
>
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT
(Not for submission under 37 CFR 1.99)

Application Number	14792094
Filing Date	2015-07-06
First Named Inventor	BRENNER, Sydney
Art Unit	1636
Examiner Name	BHAT, NARAYAN KAMESHWAR
Attorney Docket Number	CGLC-020CON5

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	9290808		2016-03-22	FODOR, Stephen P.	

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	1	WO2012148497	WO		2012-11-01	ROBINSON, et al.		

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/NARAYAN K BHAT/ 07/16/2016

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Receipt date: 06/29/2016 14792094 - GAU: 1634

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99**)	Application Number	14792094
	Filing Date	2015-07-06
	First Named Inventor	BRENNER, Sydney
	Art Unit	1636
	Examiner Name	BHAT, NARAYAN KAMESHWAR
	Attorney Docket Number	CGLC-020CON5

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EXAMINER SIGNATURE

Examiner Signature	/NARAYAN K BHAT/	Date Considered	07/16/2016

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

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

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /N.K.B/

Receipt date: 06/29/2016 14792094 - GAU: 1634

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	14792094
Filing Date	2015-07-06
First Named Inventor	BRENNER, Sydney
Art Unit	1636
Examiner Name	BHAT, NARAYAN KAMESHWAR
Attorney Docket Number	CGLC-020CON5

CERTIFICATION STATEMENT

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

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

OR

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

See attached certification statement.

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

☒ A certification statement is not submitted herewith.

SIGNATURE

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

Signature	/James S. Keddie, Reg. No. 48,920/	Date (YYYY-MM-DD)	2016-06-29
Name/Print	James S. Keddie	Registration Number	48920

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

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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14792094	BRENNER ET AL.
	Examiner	Art Unit
	NARAYAN BHAT	1634

CPC- SEARCHED		
Symbol	Date	Examiner
C12Q 1/68, C12Q 1/6874, C07H 21/02 and C40B 30/04	1/6/16	Bhat N.
Searched uisng key words in different CPC classes. See the search history in EAST.	1/6/16	Bhat N.

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Reviewed the copending applications for the inventors in PLAM and copending claims for potential ODP issues.	1/6/16	Bhat N.
Reviewed the prosecution histories of copending applications resulting in the issuance of patents.	1/6/16	Bhat N.
Reviewed the cited arts in the IDS of 10/9/15.	1/6/16	Bhat N.
searched for the topics in EAST, Pubmed and Google scholar. Search histories are enclosed.	1/6/16	Bhat N.
Updated the search for the topics in EAST. Search history is enclosed.	7/17/16	Bhat N.

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

	/NARAYAN BHAT/ Primary Examiner.Art Unit 1634

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 00660

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
14/792,094	07/06/2015	1634	800	CGLC-020CON5	19	3

CONFIRMATION NO. 9697

CORRECTED FILING RECEIPT

93726
EPA - Bozicevic Field & Francis LLP
Bozicevic, Field & Francis
1900 University Ave
Suite 200
East Palo Alto, CA 94303

Date Mailed: 07/27/2016

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

Inventor(s)

Sydney Brenner, Ely, UNITED KINGDOM;
Gi Mikawa, Great Shelford, UNITED KINGDOM;
Robert Osborne, Great Chesterford, UNITED KINGDOM;
Andrew Slatter, London, UNITED KINGDOM;

Applicant(s)

10X Genomics, Inc., Pleasanton, CA;

Power of Attorney: The patent practitioners associated with Customer Number 93726

Domestic Priority data as claimed by applicant

This application is a CON of 14/172,694 02/04/2014 PAT 9102980 *
which is a CON of 14/021,790 09/09/2013 PAT 8679756
which is a CON of 13/859,450 04/09/2013 PAT 8563274
which is a CON of 13/622,872 09/19/2012 ABN
which is a CON of 13/387,343 02/15/2012 PAT 8298767
which is a 371 of PCT/IB2010/002243 08/13/2010
which claims benefit of 61/288,792 12/21/2009
and claims benefit of 61/235,595 08/20/2009
(*)Data provided by applicant is not consistent with PTO records.

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

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Permission to Access Search Results: No

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If Required, Foreign Filing License Granted: 07/20/2015

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/792,094**

Projected Publication Date: Not Applicable

Non-Publication Request: No

Early Publication Request: No

** **SMALL ENTITY** **

Title

Compositions and Methods for Intramolecular Nucleic Acid Rearrangement

Preliminary Class

435

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

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Page 00662

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IPR2023-00955
Page 00664

Filed Electronically

CHANGE OF CORRESPONDENCE ADDRESS		
	Attorney Docket	CGLC-020CON5
	First Named Inventor	Brenner, Sydney
	Application Number	14/792,094
	Confirmation No.	9697
	Filing Date	July 6, 2015
	Group Art Unit	1634
Address to: Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Examiner Name	BHAT, NARAYAN KAMESHWAR
	Title: "Compositions and Methods for Intramolecular Nucleic Acid Rearrangement"	

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SIGNATURE OF ATTORNEY/AGENT OF RECORD

Name/Reg. No.	James S. Keddie, 48,920		
Signature	/James S. Keddie, Reg. No. 48,920/	Date	07-27-2016

Electronic Acknowledgement Receipt	
EFS ID:	26472517
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	93726
Filer:	James Samuel Keddie
Filer Authorized By:	
Attorney Docket Number:	CGLC-020CON5
Receipt Date:	27-JUL-2016
Filing Date:	06-JUL-2015
Time Stamp:	19:27:54
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Change of Address	CGLC-020CON5-Change_Corresp.pdf	20439 dfcd776d085e4422df1b1dfe2707ad202e51a2a6e1	no	1
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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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

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

PTO/AIA/82 (07-12)
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U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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POWER OF ATTORNEY TO PROSECUTE APPLICATIONS BEFORE THE USPTO

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

I hereby appoint:

■ Practitioners associated with Customer Number: 43850

OR

☐ Practitioner(s) named below (if more than ten patent practitioners are to be named, then a customer number must be used):

Name	Registration Number	Name	Registration Number

As attorney(s) or agent(s) to represent the undersigned before the United States Patent and Trademark Office (USPTO) in connection with any and all patent applications assigned only to the undersigned according to the USPTO assignment records or assignments documents attached to this form in accordance with 37 CFR 3.73(c).

Please change the correspondence address for the application identified in the attached statement under 37 CFR 3.73(c) to:

■ The address associated with Customer Number: 43850

OR

☐	Firm or Individual Name		
	Address		
	City	State	Zip
	Country		
	Telephone	Email	

Assignee Name and Address: 10X Genomics, Inc.
7068 Koll Center Parkway, Suite 401
Pleasanton, CA 94566

A copy of this form, together with a statement under 37 CFR 3.73(c) (Form PTO/AIA/96 or equivalent) is required to be Filed in each application in which this form is used. The statement under 37 CFR 3.73(c) may be completed by one of The practitioners appointed in this form, and must identify the application in which this Power of Attorney is to be filed.

SIGNATURE of Assignee of Record

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

Signature		Date	3/13/2015
Name	Matthew Murphy	Telephone	(925) 401-7495
Title	Vice President, General Counsel		

This collection of information is required by 37 CFR 1.31, 1.32 and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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

Privacy Act Statement

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

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

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: 10X Genomics, Inc.

Application No./Patent No.: 14/792,094 Filed/Issue Date: July 6, 2015

Titled: Compositions and Methods for Intramolecular Nucleic Acid Rearrangement

10X Genomics, Inc., a corporation

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

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

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

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

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

   ☐ There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. ☐ The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. ☐ The recipient, via a court proceeding or the like (*e.g.*, bankruptcy, probate), of an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

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

A. ☑ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 039159, Frame 0296, or for which a copy thereof is attached.

B. ☐ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

   1. From: _____ To: _____

      The document was recorded in the United States Patent and Trademark Office at

      Reel _____, Frame _____, or for which a copy thereof is attached.

   2. From: _____ To: _____

      The document was recorded in the United States Patent and Trademark Office at

      Reel _____, Frame _____, or for which a copy thereof is attached.

This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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

PTO/AIA/96 (08-12)
Approved for use through 01/31/2013. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

STATEMENT UNDER 37 CFR 3.73(c)

3. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

4. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

5. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

6. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

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

☑ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

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

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

Gargi Talukder — Signature

Aug 18, 2016 — Date

Gargi Talukder — Printed or Typed Name

61,368 — Title or Registration Number

Electronic Acknowledgement Receipt	
EFS ID:	26680070
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	43850
Filer:	Gargi Talukder/Sheila Gloss
Filer Authorized By:	Gargi Talukder
Attorney Docket Number:	104371-5016-US05
Receipt Date:	18-AUG-2016
Filing Date:	06-JUL-2015
Time Stamp:	14:29:20
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Power of Attorney	POA.pdf	635597 7cc73c5671666911376135 9ea9cd3d45987 f204c5	no	2
Warnings:					

Information:					
2	Assignee showing of ownership per 37 CFR 3.73	Stmt.pdf	120223 4e577fd7c80a10a20f9264 5e4d2ce34de7d7d5	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	755820	

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

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

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

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

UNITED STATES PATENT AND TRADEMARK OFFICE

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

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/792,094	07/06/2015	Sydney Brenner	104371-5016-US05

CONFIRMATION NO. 9697

43850
MORGAN, LEWIS & BOCKIUS LLP (SF)
One Market, Spear Street Tower, Suite 2800
San Francisco, CA 94105

POWER OF ATTORNEY NOTICE

OC000000085406701

Date Mailed: 08/30/2016

NOTICE REGARDING CHANGE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 08/18/2016.

- The Power of Attorney to you in this application has been revoked by the applicant. Future correspondence will be mailed to the new address of record(37 CFR 1.33).

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/khoang/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/792,094	07/06/2015	Sydney Brenner	104371-5016-US05

CONFIRMATION NO. 9697

43850
MORGAN, LEWIS & BOCKIUS LLP (SF)
One Market, Spear Street Tower, Suite 2800
San Francisco, CA 94105

POA ACCEPTANCE LETTER

OC000000085406703

Date Mailed: 08/30/2016

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 08/18/2016.

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

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/khoang/

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 9697
Sidney Brenner et al.	Examiner: Narayan Kameshwar Bhat
Application No.: 14/792,094	Art Unit: 1634
Filed: July 6, 2015	
For: COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID ARRANGEMENT	TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT

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

Commissioner:

The applicant, 10X Genomics, Inc., owner of 100 percent interest in the instant application, hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior **U.S. Patent No. 8,168,385**, as the term of said prior patent is presently shortened by any terminal disclaimer. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the applicant does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later: expires for failure to pay a maintenance fee; is held unenforceable; is found invalid by a court of competent jurisdiction; is

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00676

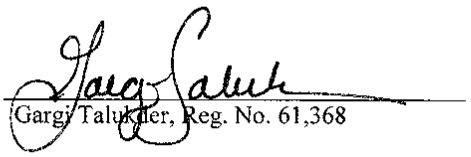
statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321; has all claims canceled by a reexamination certificate; is reissued; or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer.

Additionally, in the event that the patent term of any patent granted on the instant application is extended based on patent term adjustment under 35 U.S.C. § 154, applicant does not disclaim the extension of the patent term based on the patent term adjustment, as the term of any patent granted on the instant application has not been disclaimed beyond a specific date.

Included herewith is the terminal disclaimer fee required under 37 C.F.R. § 120(d).

The undersigned is an attorney or agent of record.

Respectfully submitted,
MORGAN, LEWIS & BOCKIUS LLP

Dated: December 22, 2016 By: 
Gargi Talukder, Reg. No. 61,368

Customer No.: 43850
Morgan, Lewis & Bockius LLP
One Market, Spear Street Tower
San Francisco, CA 94105
Telephone: (415) 442-1000
Facsimile: (415) 442-1001

DB2/ 30936205.1

PTO/SB/30 (07-14)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Request for Continued Examination (RCE) Transmittal

Address to:
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Application Number	14/792,094
Filing Date	July 6, 2015
First Named Inventor	Sidney Brenner
Art Unit	1634
Examiner Name	Narayan, Kameshwar Bhat
Attorney Docket Number	104371-5016-US05

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 compy with the requirements of 35 U.S.C 371, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO on page 2.)

1. Submission required under 37 CFR 1.114 Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

   a. ☐ 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.

      i. ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____

      ii. ☐ Other _____

   b. ☑ Enclosed

      i. ☑ Amendment/Reply

      ii. ☐ Affidavit(s)/ Declaration(s)

      iii. ☐ Information Disclosure Statement (IDS)

      iv. ☐ Other _____

2. Miscellaneous

   a. ☐ 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)

   b. ☐ Other _____

3. Fees The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.

   a. ☑ The Director is hereby authorized to charge the following fees, any underpayment of fees, or credit any overpayments, to Deposit Account No. 50-0310 .

      i. ☑ RCE fee required under 37 CFR 1.17(e)

      ii. ☑ Extension of time fee (37 CFR 1.136 and 1.17)

      iii. ☐ Other _____

   b. ☐ Check in the amount of $ _____ enclosed

   c. ☐ Payment by credit card (Form PTO-2038 enclosed)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Signature	/Gargi Talukder/	Date	December 22, 2016
Name (Print/Type)	Gargi Talukder, Ph.D.	Registration No.	61,368

CERTIFICATE OF MAILING OR TRANSMISSION

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450 or facsimile transmitted to the U.S. Patent and Trademark Office on the date shown below.

Signature	/Leonor Rivera-Huerta/		
Name (Print/Type)	Leonor Rivera-Huerta	Date	December 22, 2016

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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

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Instruction Sheet for RCEs

(not to be submitted to the USPTO)

NOTES:

An RCE is not a new application, and filing an RCE will not result in an application being accorded a new filing date.

Filing Qualifications:

The application must be a utility or plant application filed on or after June 8, 1995. The application cannot be a provisional application, a utility or plant application filed before June 8, 1995, an international application that does not comply with the requirements of 35 U.S.C. 371, a design application, or a patent under reexamination. See 37 CFR 1.114(e). An international application does not comply with the requirements of 35 U.S.C. 371 until the requirements under 35 U.S.C. 371(c), including the requirement for the inventor's oath or declaration under 35 U.S.C. 371(c)(4), have been complied with.

Filing Requirements:

Prosecution in the application must be closed. Prosecution is closed if the application is under appeal, or the last Office action is a final action, a notice of allowance, or an action that otherwise closes prosecution in the application (e.g., an Office action under *Ex parte Quayle*). See 37 CFR 1.114(b).

A submission and a fee are required at the time the RCE is filed. If reply to an Office action under 35 U.S.C. 132 is outstanding (e.g., the application is under final rejection), the submission must meet the reply requirements of 37 CFR 1.111. If there is no outstanding Office action, the submission can be an information disclosure statement, an amendment, new arguments, or new evidence. See 37 CFR 1.114(c). The submission may be a previously filed amendment (e.g., an amendment after final rejection).

WARNINGS:

Request for Suspension of Action:

All RCE filing requirements must be met before suspension of action is granted. A request for a suspension of action under 37 C FR 1.103(c) does not satisfy the submission requirement and does not permit the filing of the required submission to be suspended.

Improper RCE will NOT toll Any Time Period:

Before Appeal - If the RCE is improper (e.g., prosecution in the application is not closed or the submission or fee has not been filed) and the application is not under appeal, the time period set forth in the last Office action will continue to run and the application will be abandoned after the statutory time period has expired if a reply to the Office action is not timely filed. No additional time will be given to correct the improper RCE.

Under Appeal - If the RCE is improper (e.g., the submission or the fee has not been filed) and the application is under appeal, the improper RCE is effective to withdraw the appeal. Withdrawal of the appeal results in the allowance or abandonment of the application depending on the status of the claims. If there are no allowed claims, the application is abandoned. If there is at least one allowed claim, the application will be passed to issue on the allowed claim(s). See MPEP 1215.01.

See MPEP 706.07(h) for further information on the RCE practice.

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00679

Privacy Act Statement

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

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

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PTO/AIA/22 (03-13)
Approved for use through 7/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)

Docket Number (Optional): 104371-5016-US05

Application Number: 14/792,094

Filed: July 6, 2015

For: Compositions and Methods for Intramolecular Nucleic Acid Rearrangement

Art Unit: 1634

Examiner: BHAT, NARAYAN KAMESHWAR

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

	Fee	Small Entity Fee	Micro Entity Fee	
☐ One month (37 CFR 1.17(a)(1))	$200	$100	$50	$
☑ Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$ 300
☐ Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$
☐ Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$
☐ Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

☑ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 50-0310.

☑ Payment made via EFS-Web.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant.

☑ attorney or agent of record. Registration number 61,368.

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____.

Gargi Talukder — Signature — December 22, 2016 — Date

Gargi Talukder, Ph.D. — Typed or printed name — 415-442-1000 — Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☑ * Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.136(a). The information is required to obtain or retain a benefit by the public, which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 6 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: Mail Stop PCT, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 9697
Sidney Brenner et al.	Examiner: Narayan Kameshwar Bhat
Application No.: 14/792,094	Art Unit: 1634
Filed: July 6, 2015	
For: COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID ARRANGEMENT	RESPONSE TO FINAL OFFICE ACTION

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

Commissioner:

In response to the Final Office Action mailed July 25, 2016, please enter the following amendments and remarks:

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

Remarks/Arguments begin on page **5** of this paper.

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Amendments to the Claims:

This listing of claims will replace all prior versions, and listings of claims in the application:

Listing of Claims:

1-39 (Cancelled)

40. (New) A method of analyzing nucleic acids, the method comprising:

(a) providing a first plurality of segments of nucleic acid molecules from a contiguous region of a genome from a first single isolated cell, wherein each of the first plurality of segments is appended to a first multiplex identifier (MID) sequence to form first appended segments;

(b) providing a second plurality of segments of nucleic acid molecules from a contiguous region of a genome from a second single isolated cell, wherein each of the second plurality of segments is appended to a second MID sequence to produce second appended segments, and the first MID sequence and the second MID sequence are different from each other;

(c) sequencing the first appended segments and the second appended segments to provide a plurality of sequences,

wherein each sequence comprises (i) nucleotide sequence of at least a portion of a segment and (ii) an associated MID sequence, and wherein the MID sequences uniquely identify the segments with a single isolated cell.

41. (New) The method of claim 40, wherein the method further comprises amplifying the first appended segments and the second appended segments before the sequencing step (c).

42. (New) The method of claim 40, wherein the nucleic acid molecules are selected from DNA and RNA.

43. (New) The method of claim 42, wherein the nucleic acid molecules comprise mRNA.

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44. (New) The method of claim 40, wherein the MID sequences are appended to the segments through ligation.

45. (New) The method of claim 40, wherein the MID sequences are appended to the segments by performing PCR or linear amplification of the plurality of nucleic acids using an adapter sequence comprising the MID sequence and an amplification primer.

46. (New) The method of claim 40, wherein the sequences of the segments are attributed to different isolated cells based on the associated MID sequence.

47. (New) The method of claim 40, wherein the MID sequences further comprise a sequencing primer sequence.

48. (New) The method of claim 40, wherein the MID sequences are from 2 to 100 bases in length.

49. (New) The method of claim 40, wherein the segments of (a) and (b) are in the range of 100 to 5,000 bp in length.

50. (New) The method of claim 40, wherein the segments of (a) and (b) are 400 bp or less in length.

51. (New) The method of claim 40, wherein the method further comprises providing a third plurality of segments of nucleic acid molecules from a contiguous region of a genome from a third single isolated cell, wherein each of the third plurality of segments is appended to a third MID sequence to produce third appended segments, and the third MID sequence is different from the first MID sequence and the second MID sequence.

52. (New) The method of claim 40, wherein the contiguous region is greater than 2 kb in length.

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53. (New) A method of analyzing a plurality of contiguous genomic sequences, comprising:

(a) providing a sample comprising the plurality of contiguous genomic sequences, wherein the plurality of contiguous genomic sequences are from a plurality of single isolated cells;

(b) tagging a plurality of fragments of the contiguous genomic sequences with a multiplex identifier (MID) sequence such that fragments of a first contiguous nucleic acid sequence share a common MID sequence that is different from an MID sequence of fragments of a second contiguous nucleic acid sequence; and

(c) sequencing the plurality of fragments and MID sequences to identify fragments of the contiguous sequence.

54. (New) The method of claim 53, wherein the first contiguous nucleic acid sequence is from a first single isolated cell and the second contiguous nucleic acid sequence is from a second single isolated cell.

55. (New) The method of claim 53, wherein sequences of the fragments are attributed to different single isolated cells based on the associated MID sequence.

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Remarks/Arguments

Applicants have not dedicated or abandoned any unclaimed subject matter and have not acquiesced to any rejections made by the Patent Office. Applicants reserve the right to pursue prosecution of any presently excluded claim embodiments in future continuation and/or divisional applications.

Status of the Claims

Claims 40-55 are newly added and under consideration in this application. The newly added claims are supported throughout the specification as filed, for example, see page 7 and pages 16-20 of the application as filed.

Applicants respectfully request entry of the claims into the record.

Response to Office Action

Claim Objections

Claim 28 is objected to because it is dependent from canceled claim 23. Claim 28 is canceled in the currently pending claims, thus rendering this objection moot.

Claim Rejections – 35 USC § 102 and § 103

The previous rejections of the claims under § 102 as being allegedly anticipated by Braverman et al. (US 2009/0105959 (hereinafter "Braverman") and under § 103 as allegedly being obvious over Braverman in view of Bosnes, WO 03/062462 (hereinafter "Bosnes") have been maintained. Applicants respectfully traverse these rejections.

In order to clarify the claimed invention, Applicants have prepared a new set of claims. Applicants submit that Braverman fails to disclose all elements of the claimed invention as clarified in this set of claims. In particular, Braverman fails to disclose a method in which each of a first plurality of segments from a first isolated cell is appended to a first multiplex identifier (MID) and each of a second plurality of segments from a second isolated cell are appended to a

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second MID, as is recited in independent claim 40. In addition, Braverman fails to provide a method in which a plurality of fragments of contiguous genomic sequences are tagged with a MID sequence such that fragments of a first contiguous nucleic acid sequence share a common MID sequence that is different from an MID sequence of fragments of a second contiguous nucleic acid sequence, as is recited in independent claim 53. Furthermore, Braverman fails to disclose methods in which segments appended to or tagged with MIDs can be attributed to their single cell of origin. As such, Braverman fails to disclose or suggest all elements of the claimed invention and thus cannot support a rejection for anticipation.

Bosnes is cited solely for its description of methods of analyzing mRNA and thus fails to rectify the above-discussed deficiencies in Braverman. As such, the combined teachings of Braverman and Bosnes fail to render the presently claimed invention obvious.

For at least the foregoing reasons, Applicants respectfully request that all rejections under § 102 and § 103 be withdrawn.

Claim Rejections – Double Patenting

Claims 21, 24, and 26-39 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-12 of U.S. Patent No. 8,168,385 in view of Braverman et al., and Bosnes.

Claims 21, 24, and 26-39 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-13 of U.S. Patent No. 8,318,433 in view of Braverman and Bosnes.

Without conceding to the propriety of these rejections and solely to move this application toward allowance, Applicants herewith file terminal disclaimers over the above-referenced patents.

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CONCLUSION

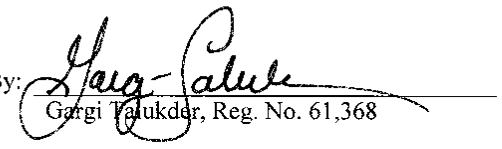
All the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn.

Applicants believe that a full and complete reply has been made to the outstanding Office Action, and as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided below.

Except for issue fees payable under 37 C.F.R. § 1.18, the Commissioner is hereby authorized by this paper to charge any necessary fees during the entire pendency of this application including fees due under 37 C.F.R. §§ 1.16 and 1.17, which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-0310 (Order No.: 104371-5016-US05).

Prompt and favorable consideration of this Amendment and Response is respectfully requested.

Respectfully submitted,
MORGAN, LEWIS & BOCKIUS LLP

Dated: December 22, 2016 By: 
Gargi Talukder, Reg. No. 61,368

Customer No.: 43850
Morgan, Lewis & Bockius LLP
One Market, Spear Street Tower
San Francisco, CA 94105
Telephone: (415) 442-1000
Facsimile: (415) 442-1001

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 9697
Sidney Brenner et al.	Examiner: Narayan Kameshwar Bhat
Application No. 14/792,094	Art Unit: 1634
Filed: July 6, 2015	
For: COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID ARRANGEMENT	TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT

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

Commissioner:

The applicant, 10X Genomics, Inc., owner of 100 percent interest in the instant application, hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior **U.S. Patent No. 8,318,433**, as the term of said prior patent is presently shortened by any terminal disclaimer. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the applicant does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later: expires for failure to pay a maintenance fee; is held unenforceable; is found invalid by a court of competent jurisdiction; is

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statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321; has all claims canceled by a reexamination certificate; is reissued; or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer.

Additionally, in the event that the patent term of any patent granted on the instant application is extended based on patent term adjustment under 35 U.S.C. § 154, applicant does not disclaim the extension of the patent term based on the patent term adjustment, as the term of any patent granted on the instant application has not been disclaimed beyond a specific date.

Included herewith is the terminal disclaimer fee required under 37 C.F.R. § 120(d).

The undersigned is an attorney or agent of record.

Respectfully submitted,
MORGAN, LEWIS & BOCKIUS LLP

Dated: December 22, 2016 By: 
Gargi Talukder, Reg. No. 61,368

Customer No.: 43850
Morgan, Lewis & Bockius LLP
One Market, Spear Street Tower
San Francisco, CA 94105
Telephone: (415) 442-1000
Facsimile: (415) 442-1001

DB2/ 30936273.1

Electronic Patent Application Fee Transmittal

Application Number:	14792094
Filing Date:	06-Jul-2015
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Filer:	Gargi Talukder/Leonor Rivera-Huerta
Attorney Docket Number:	104371-5016-US05

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:				
PUBL. FEE- EARLY, VOLUNTARY, OR NORMAL	1504	1	0	0
PROCESSING FEE, EXCEPT PROV. APPLS.	2830	1	70	70
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 2 months with $0 paid	2252	1	300	300
Miscellaneous:				
RCE- 1st Request	2801	1	600	600
STATUTORY OR TERMINAL DISCLAIMER	2814	2	160	320
		Total in USD ($)		**3290**

Electronic Acknowledgement Receipt	
EFS ID:	27885111
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	43850
Filer:	Gargi Talukder/Leonor Rivera-Huerta
Filer Authorized By:	Gargi Talukder
Attorney Docket Number:	104371-5016-US05
Receipt Date:	22-DEC-2016
Filing Date:	06-JUL-2015
Time Stamp:	19:07:50
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$3290
RAM confirmation Number	122316INTEFSW00006963500310
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	TrackOne Request	104371_5016US05_Track_1_Request.pdf	335899 [illegible]	no	1
Warnings:					
Information:					
2	Request for Continued Examination (RCE)	104371_5016US05_RCE.pdf	839149 [illegible]	no	3
Warnings:					
This is not a USPTO supplied RCE SB30 form.					
Information:					
3	Extension of Time	104371_5016US05_EOT.pdf	288822 [illegible]	no	1
Warnings:					
Information:					
4		104371_5016US05_Response_to_Final_Office_Action.pdf	842216 [illegible]	yes	7

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment Submitted/Entered with Filing of CPA/RCE	1	1
Claims	2	4
Applicant Arguments/Remarks Made in an Amendment	5	7

Warnings:
Information:

5	Terminal Disclaimer Filed	104371_5016US05_TD_over_US8168385.pdf	270993 [illegible]	no	2
Warnings:					
Information:					
6	Terminal Disclaimer Filed	104371_5016US05_TD_over_US8318433.pdf	262359 [illegible]	no	2
Warnings:					
Information:					
7	Fee Worksheet (SB06)	fee-info.pdf	41171 [illegible]	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	2880609		

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

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

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

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

Doc Code: TRACK1.REQ
Document Description: TrackOne Request

PTO/AIA/424 (04-14)

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

First Named Inventor:	Sidney Brenner	Nonprovisional Application Number (if known):	14/792,094
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement		

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 /Gargi Talukder/	Date December 22, 2016
Name (Print/Typed) Gargi Talukder, Ph.D.	Practitioner Registration Number 61,368

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.

PTO/SB/06 (09-11)
Approved for use through 1/31/2014 OMB 0651-0032
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/792,094	Filing Date 07/06/2015	☐ 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), (l), 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	12/22/2016	(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))	* 16	Minus	** 20	= 0	X $40 =	0
	Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	= 0	X $210 =	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.

LIE
William Phillips

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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MORGAN, LEWIS & BOCKIUS LLP (SF)
One Market, Spear Street Tower, Suite 2800
San Francisco CA 94105

MAILED
JAN 10 2017
OFFICE OF PETITIONS

Doc Code: TRACK1.GRANT

Decision Granting Request for Prioritized Examination (Track I or After RCE)	Application No.: 14/792,094

1. THE REQUEST FILED December 22, 2016 IS **GRANTED.**

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

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

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

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

Telephone inquiries with regard to this decision should be directed to Brian W. Brown at 571-272-5338.

/Brian W. Brown/
[*Signature*]

Petitions Examiner, Office of Petitions
(Title)

U.S. Patent and Trademark Office
PTO-2298 (Rev. 02-2012)

Application Number	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14792094	BRENNER ET AL.

Document Code - DISQ	Internal Document – DO NOT MAIL

TERMINAL DISCLAIMER	☒ APPROVED	☐ DISAPPROVED
Date Filed: 12/22/2016	This patent is subject to a Terminal Disclaimer	

Approved/Disapproved by:	
2 TD's	/T.J./ Art Unit OCRU

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/792,094	07/06/2015	Sydney Brenner	104371-5016-US05	9697

43850 7590 02/01/2017

MORGAN, LEWIS & BOCKIUS LLP (SF)
One Market, Spear Street Tower, Suite 2800
San Francisco, CA 94105

EXAMINER
BHAT, NARAYAN KAMESHWAR

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
02/01/2017	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):

sfipdocketing@morganlewis.com
donald.mixon@morganlewis.com

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00700

Office Action Summary	Application No. 14/792,094	Applicant(s) BRENNER ET AL.	
	Examiner NARAYAN BHAT	Art Unit 1634	AIA (First Inventor to File) Status No

-- 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 *12/22/16*.

☐ 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) *40-55* is/are pending in the application.

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

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *40-55* is/are rejected.

8)☒ Claim(s) *53* is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

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

Application Papers

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

11)☒ The drawing(s) filed on *7/6/15* 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: _____.

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00701

Continued Examination under 37 CFR 1.114

1. 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 22, 2016 has been entered.

Track1 Request – Granted

2. Track1 request filed by the applicant on December 22, 2016 has been granted by the office on January 10, 2017. Therefore instant application has been examined on a priority basis. To maintain its status applicant is required to follow the requirements listed in the Track1 grant document.

3. The present application is being examined under the pre-AIA first to invent provisions. The instant application is a continuation of 14/172,694, now U.S. Patent 9,102,980, which is a continuation of 14/021,790, now U.S. Patent 8,679,756, which is a continuation of 13/859,450, now U.S. Patent 8,563,274, which is a continuation of 13/622,872, now abandoned, which is a continuation of 13/387,343, now U.S. Patent 8,298,767, which is a national stage entry of PCT/IB2010/002243, claiming priority from provisional application 61288792, filed on 12/21/2009 and from provisional application 61235595, filed on 08/20/2009.

This office action is in response to the papers filed on December 22, 2016.

Claim Status

3. In the claim listing of December 22, 2016 claims 40-55 are pending in this application and are under prosecution. Claims 40-55 are new. Claims 1-39 are canceled. Claims have been reviewed and entered. As discussed below, the limitation of claim 53 of "a first contiguous nucleic acid sequence share a common MID sequence that is different from an MID sequence of fragments of second contiguous nucleic acid sequence" introduces a new matter (new matter is underlined by the examiner).

Withdrawn Objection, Rejections and Response to the Remarks

4. The previous objection of claim 28 has been withdrawn in view of cancellation of said claim. (Remarks, pg. 5).

5. The previous rejection under pre-AIA 35 USC 102(b) as being anticipated by Braverman and rejection under pre-AIA 35USC 103(a) as being unpatentable over Braverman in view of Bosnes have been withdrawn in view of cancellation of said claims.

The arguments regarding the teachings of Braverman as they pertain to the rejection made in this office action have been fully considered (Remarks, pgs. 5 and 6). They are not persuasive for the following reasons.

The arguments are directed to Braverman fails to disclose a method in which each of a first plurality of segments from a first isolated cell is appended to a first multiplex identifier (MID) and each of a second plurality of segments from a second isolated cell are appended to a second MID, as is recited in independent claim 40. In

addition, Braverman fails to provide a method in which a plurality of fragments of contiguous genomic sequences are tagged with a MID sequence such that fragments of a first contiguous nucleic acid sequence share a common MID sequence that is different from an MID sequence of fragments of a second contiguous nucleic acid sequence, as is recited in independent claim 53. Furthermore, Braverman fails to disclose methods in which segments appended to or tagged with MIDs can be attributed to their single cell of origin. As such, Braverman fails to disclose or suggest all elements of the claimed invention and thus cannot support a rejection for anticipation (Remarks, pg. 6).

The arguments regarding the subject matter of claims 40 and 53 as being anticipated by Braverman are moot because of withdrawn rejection necessitated by cancellation of previous claims and addition of new claims.

With regard to claimed invention being obvious over Braverman in view of Drmanac (new art, see below) are not persuasive because new claim 40 as recited require providing two single isolated cells, which could be the same but processed independently, wherein nucleic acid template (i.e., a contiguous region of the genome) subjected to an amplification thereby producing a plurality of segments of nucleic acids, wherein said segments from each single cells are appended with a single cell specific unique identifier (UID) or referred also as multiplex identifier (MID), pooling them and sequencing them and sequence data for segments from single cells de-convoluted to identify the sequence of the MID sequence and their association with each of the two cells. Claim 53 has additional "common sequence" in MID.

Braverman teaches the steps of claims 40 and 53 including the analyzing nucleic acid from a single cell and also teaches analyzing nucleic acid template from multiple samples and sequencing the segments of the nucleic acid template (i.e., contiguous region of the genome) from each sample and identifying the segments from each sample associated with specific multiplex identifier from each sample that includes a cell. Braverman does not specifically teach that the nucleic acid segments from at least two isolated single cells. The teachings of Drmanac are specifically applied for the teachings of analyzing nucleic acids using the tagging (i.e., using multiplex identifier) method similar to the Braverman, wherein the nucleic acids are derived from two aliquots (i.e., samples) wherein each aliquot comprises a single isolated cell, thereby meeting the limitations of two isolated single cells.

The unique identifier element (also referred as multiplex identifier) tagged to each nucleic template comprising plurality of PCR amplified segments from each sample and identifying each sample origin associated with the unique multiplex identifier sequence of Braverman meets the limitations of the multiplex identifier. Furthermore, Braverman teaches key element 207 associated with each multiplex identifier, which meets the limitation of sharing common sequence associated with different multiplex identifiers (limitation of claim 53).

The routine, well established and conventional method of identifying the polynucleotides in each sample from the pooled samples using unique tags for each sample nucleic acids as applied for the rejection of claims 40 and 53 are further evidenced by Gillevet (WO 2008/061193; Fig. 1 and pg. 17, paragraph 3).

Therefore it maintained that the limitations of claims 40-55 are obvious over Braverman in view of Drmanac as further evidenced by Gillevet.

7. The previous ODP rejection of claims 21, 24 and 26-39 over claims 1-12 of '385 patent or over claims of '433 patent has been withdrawn in view of filing the terminal disclaimer on 12/22/16 and its acceptance by the office on 1/22/17.

Claim Objections

8. Claim 53 is objected to because of the following informalities. The recitation of "an MID sequence" in step 'b' lines 3 and 4 has been objected because of minor typographical error. It is suggested to use the phrase "a MID sequence" instead for consistency. Appropriate correction is required.

Claim Rejections - 35 USC § 112-first paragraph

8. 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.

9. 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.

10. Claims 40-55 are 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. eMPEP 2163.06 notes, "If new matter is added to the claims, the examiner should reject the claims under 35 U.S.C. 112, first paragraph written description requirement (In re Rasmussen, 650 F.2d 1212, 211 USPQ 323 (CCPA 1981)."

In the instant case, the recitations of "a genome from a first single isolated cell" and "a genome from a second single isolated cell" in claim 40 and "a first contiguous nucleic acid sequence share a common MID sequence that is different from an MID sequence of fragments of a second contiguous nucleic acid sequence" in claim 53 represents a new matter (New matter is underlined by the Examiner). Applicant has cited support for the new claims in the specification pages 7 and 16-20 (Remarks, pg. 5, paragraph 2). However, review of the cited pages in the instant specification does not support the "isolated single cell" and "common MID sequence". Also, review of the instant figure 12 shows the presence of a separate "common" sequence among three different nucleic acid segment and thus appears to be the "same" common sequence rather than different "common sequence". Pending further clarification, the "common MID sequence" introduces the new matter.

Also, "the written description requirement prevents an applicant from claiming subject matter that was not adequately described in the specification as filed. New or amended claims which introduce elements or limitations which are not supported by the as-filed disclosure violate the written description requirement. See, e.g., In re Lukach, 442 F.2d 967, 169 USPQ 795 (CCPA 1971) (subgenus range was not supported by generic disclosure and specific example within the subgenus range); In re Smith, 458 F.2d 1389, 1395, 173 USPQ 679, 683 (CCPA 1972) (a subgenus is not necessarily described by a genus encompassing it and a species upon which it reads)." (MPEP § 2163).

Since Applicant has not identified specific citations of support in the instant specification or in claims as originally filed for the new matter, Applicant is required to cancel the new matter in the reply to this Office Action or identify the new matter in the specification as originally filed.

11. Claims 41-52 and 54-55 are rejected under 35 USC first paragraph because they are dependent from claims 1 and 53.

Claim Rejections - 35 USC § 112-second paragraph

12. 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.

13. 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.

14. Claims 53-55 are 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.

15. Claim 53 is indefinite over the recitation of "a first contiguous nucleic acid sequence share a common MID sequence that is different from a MID sequence of fragments of second contiguous nucleic acid sequence" in step 'b' it is unclear how sharing sequences is different. Further amendment or clarification is required.

16. Claims 54 and 55 are indefinite because they are dependent from claim 53.

Claim Rejections - 35 USC § 103

17. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

18. Claims 40-55 are rejected under pre-AIA 35 U.S.C. 102(a) and 102(e) as being anticipated by Braverman et al (US 2009/0105959, cited in the previous office action) in view of Drmanac (US 2007 /0072208 published Mar. 29, 2007). The routine, well established and conventional method of identifying the polynucleotides in each sample from the pooled samples using unique tags for each sample nucleic acids as applied for the rejection of claims 40 and 53 are further evidenced by Gillevet (WO 2008/061193 published May 22, 2008).

Claim interpretation: The steps of claim 40 has been interpreted as providing multiple samples that includes a first single isolated cell and a second single isolated

cell, wherein nucleic acid template (i.e., a contiguous nucleic acid from genome) produced by PCR amplification from each sample (i.e., plurality of segments) is appended with a sample specific unique identifier (UID) as also referred as multiplex identifier (MID), pooling the appended segments and sequencing them and sequence data for each template nucleic acid (i.e., plurality of segments) is de-convoluted to identify the sequence composition of coupled UID or MID sequences and its association with the sample of origin.

The steps of claims 53-55 has been interpreted to encompass the steps of claim 40 except for sharing a common sequence.

It is noted that the recitation of the first single isolated cell and the second single isolated cell does not exclude the first and the second being the same cell but are appended with different multiplex identifier.

Braverman and Drmanac teach a method for analyzing nucleic acid from a sample and therefore are analogous arts. Braverman teaches the steps of claims 40 and 53 including the analyzing nucleic acid from a single cell and also teaches analyzing nucleic acid template from multiple samples and sequencing the segments of the nucleic acid template (i.e., contiguous region of the genome) from each sample and identifying the segments from each sample associated with specific multiplex identifier from each sample that includes a cell. Braverman does not specifically teach that the nucleic acid segments from at least two isolated single cells. The teachings of Drmanac are specifically applied for the teachings of analyzing nucleic acids using the tagging method similar to the Braverman, wherein the nucleic acids are derived from two

aliquots (i.e., samples) wherein each aliquot comprises a single isolated cell, which are useful for more accurate pathogen diagnostic for health and biodefense applications.

Regarding claims 40 and 53-55, Braverman method of analyzing nucleic acids, comprising providing a plurality of nucleic acid segments from a nucleic acid template (i.e., contiguous region of the genome) from a single cell source (paragraph 0077) and tagging (i.e., associating) the plurality of segments from the single cell source with an unique identifier (UID) element also referred as multiplex identifier (MID; paragraph 0003) and determining a nucleotide sequence of the segments and attached MID sequences, wherein the MID sequences uniquely identify the single cell source (Fig. 2 and paragraphs 0003, 0077, 0082-0088). Braverman also teaches that the nucleic acid templates (i.e., the contiguous region of the genome) is amplified by PCR amplification from each sample, thereby generating plurality of segments of nucleic acids and appended with a sample specific unique identifier (i.e., UID or MID), pooling the appended segments and sequencing them and the sequence data for each template nucleic acid is de-convoluted to identify the sequence composition of coupled UID element and its association with sample of origin (paragraphs 0046, 0047 and 0077).

As discussed above, though Braverman teaches that the DNA is from single cell but does not specifically teach that the nucleic acid segments are derived from two isolated single cells. However, providing the nucleic acid segments from genomic DNA from two isolated single cells were known in the art at the time the claimed invention was made as taught by Drmanac, who teaches a method for analyzing mixtures of bacterial cells genomes, wherein each aliquot comprises a single cell analyzing plurality

of single cells using the tagging method (Fig. 1 and paragraphs 0009 and 0043) similar to Braverman and further teaches the step for tracking the nucleic acid using oligonucleotide tag (i.e., identifier) for each polynucleotide (paragraph 0105). Drmanac also teaches analyzing plurality of single cell nucleic acid DNA separately allows for more accurate pathogen diagnostic for health or biodefense applications including the changes associated with the single cell (paragraph 0043), thus providing motivation to one of ordinary skill in the art to include the step of analyzing nucleic acid from a plurality of individual single cells of Drmanac in the method of Braverman. The routine, well established and conventional method of identifying the polynucleotides in each sample from the pooled samples using unique tags for each sample nucleic acids as applied for the rejection of claims 40 and 53 are further evidenced by Gillevet (Fig. 1 and pg. 17, paragraph 3).

One having ordinary skill in the art would also recognize that while combining the steps of Braverman with the step of Drmanac some routine optimization is necessary which is within the skills of one having ordinary skill in the art and motivation to include the missing step comes from Drmanac and not gleaned from the instant specification.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the step of analyzing nucleic acid from a plurality of individual single cells of Drmanac in the method of Braverman with a reasonable expectation of success with the expected benefit of more accurate pathogen diagnosis and changes associated with each single cell. An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves

including the two individual single cells for analysis which is routinely practiced in the art and therefore not novel.

The teachings of Braverman in view of Drmanac regarding dependent claims 41-52 are discussed below.

Regarding claim 41, Braverman teaches an amplification of plurality of nucleic acid attached to the MID sequence before determining the nucleic acid sequence (paragraph 0088).

Regarding claims 42 and 43 Braverman teaches that the nucleic acids are DNA or mRNA (paragraph 0026).

Regarding claim 44, Braverman teaches that the tagging step comprises ligating a sequence comprising the MID sequences to the plurality nucleic acids (paragraphs 0097).

Regarding claims 45 and 47, Braverman teaches that the tagging step comprises performing PCR of the plurality of nucleic acids using an adapter sequence comprising the MID sequence and an amplification primer (Fig. 2 and paragraphs 0088 and 0096-0099).

Regarding claim 46, Braverman in view of Drmanac teaches providing a plurality of nucleic acids from each of a plurality of single cell sources; the attaching step comprises attaching different MID sequences to the nucleic acids from each of the plurality of single cell sources; and wherein nucleic acid sequences are attributed to different individual cell sources based upon the MID sequences attached in the

attaching step and a sequencing primer sequence (Braverman, Fig. 2 and paragraphs 0085-0088).

Regarding claim 48, Braverman teaches that the MID sequences are 10-mer in length, which is within the range of 2 to 100 bases in length and at least 144 different MID sequences (paragraph 0092).

Regarding claims 49 and 50, Braverman teaches that the nucleic acid segments are about 200 to 300 bp in length (paragraph 0047), which is in the claimed range of 100 to 5000 bp.

Regarding claim 51, the step of providing third isolated single cell and the nucleic acid appended with third MID sequence is mere duplication and therefore not novel especially when Braverman teaches multiplexed sample comprise about 384 samples (paragraph 0077), wherein each sample is identified by an unique identifier (paragraph 0077).

Regarding claim 52, Drmanac teaches that nucleic acid template is in the range of 10-30 kb (paragraph 0023), which is greater than 2 kb in length.

It is further noted that in KSR, the Supreme Court particularly emphasized "the need for caution in granting a patent based on the combination of elements found in the prior art," (USPQ2d at 1395), and reaffirmed principles based on its precedent that "[t]he combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results."

In the instant case, the steps of claims 40-55 are a combination of known steps which are well understood, routine, conventional steps already engaged in by the

scientific community for improving the sensitivity, specificity of the nucleic acid analysis and were known in the art at the time the claimed invention was made. As discussed above, Braverman in view of Drmanac teaches the claimed steps and combined as suggested above would arrive at the claimed invention. Thus the steps of pending claims when viewed as whole, add nothing significant beyond the sum of the steps taken from Braverman and Drmanac. Therefore the steps of claims 40-55 do not amount to "significantly more" and thus obvious improvement over Braverman.

Double Patenting

19. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the claims at issue are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); In re Vogel, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l) (1) - 706.02(l) (3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/forms/. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all

requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

20. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-12 of U.S. Patent No. 8,679,756 in view of Drmanac (US 2007 /0072208 published Mar. 29, 2007) for the following reasons. The routine, well established and conventional method of identifying the polynucleotides in each sample from the pooled samples using unique tags for each sample nucleic acids as applied for the rejection of claims 40 and 53 are further evidenced by Gillevet (WO 2008/061193 published May 22, 2008).

Regarding instant independent claims 40 and 53, claims 1, 2 and 11 of '756 patent discloses a method comprising: a) obtaining a composition comprising a mixture of: i. a first plurality of different segments of a first nucleic acid molecule of a contiguous region in genomic DNA, wherein said contiguous region in genomic DNA is represented by a plurality of nucleic acid molecules, and wherein each of said first plurality of segments is appended to a first multiplex identifier (MID) sequence; and ii. a second plurality of different segments of a second nucleic acid molecule of said contiguous region, wherein each of said second plurality of segments is appended to a second MID sequence and said first MID sequence and said second MID sequence are different, wherein, in said composition, all of the segments of each of the nucleic acid molecules of the contiguous region comprises a unique MID sequence that uniquely identifies each of the nucleic acid molecules; and b) sequencing said first plurality of segments and said a second plurality of segments to provide a plurality of sequences; wherein each

sequence comprises: (i) the nucleotide sequence of at least a portion of a segment and (ii) an associated MID sequence, wherein said genomic DNA is genomic DNA from a virus or a bacterium, wherein defining a linkage relationship between said sequences using the MID sequence that is associated with each of the sequences, wherein sequences that comprise the first MID sequence are linked and sequences that comprise the second MID sequence are linked (common steps between instant claims 40 and 53 are underlined by the examiner). One having ordinary skill in the art would recognize that genomic DNA from a bacterium amounts of a genome from a single isolated cell. One having ordinary skill in the art would also recognize that providing a genome from a second isolated cell would be a duplication of the steps of '756 patent because instant claims as recited merely require analysis of the first and second appended segments and not linking the sequence to the first and the second isolated single cell.

However, claims of '756 patent do not disclose nucleic acid segments are from first and second cell. However, providing the genomic DNA from two bacteria was known in the art at the time the claimed invention was made as taught by Drmanac, who teaches a method for analyzing mixtures of bacterial genomes, aliquots may be made so that each aliquot contains one cell type, or a maximum of one cell (paragraph 0043) and further teaches method for tracking the nucleic acid using oligonucleotide tag (i.e., identifier) for tracking each polynucleotide (paragraph 0105). Drmanac also teaches analyzing single cell nucleic acid DNA allows for more accurate pathogen diagnostic for health or biodefense applications (paragraph 0043), thus providing motivation to one of

ordinary skill in the art to include the step of analyzing nucleic acid from a plurality of individual single cell of Drmanac in the method of '756 patent. The routine, well established and conventional method of identifying the polynucleotides in each sample from the pooled samples using unique tags for each sample nucleic acids as applied for the rejection of claims 40 and 53 are further evidenced by Gillevet (Fig. 1 and pg. 17 and paragraph 3).

One having ordinary skill in the art would also recognize that while combining the steps of '756 patent with the step of Drmanac some routine optimization is necessary which is within the skills of one having ordinary skill in the art and motivation to include the missing step comes from Drmanac.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the step of analyzing nucleic acid from a plurality of individual single cell of Drmanac in the method of '756 patent with a reasonable expectation of success with the expected benefit of more accurate pathogen diagnosis. An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including the two single cell analysis which is routinely done in the art and therefore the steps of instant claims 40 and 53 are not patentably distinct from claims of '756 patent in view of Drmanac.

With regard to instant dependent claims 41-52, 54 and 55, claims of '756 patent in view of Drmanac disclose the steps and therefore are not patentably distinct.

21. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-11 of U.S. Patent No. 8,563,274 in view of Drmanac (US 2007 /0072208 published Mar. 29, 2007) for the following reasons. The routine, well established and conventional method of identifying the polynucleotides in each sample from the pooled samples using unique tags for each sample nucleic acids as applied for the rejection of claims 40 and 53 are further evidenced by Gillevet (as cited above).

Regarding instant claims 40 and 53, claims 1 and 10 of '274 patent is drawn to a composition comprising a mixture of: a) a first plurality of different segments of a first nucleic acid molecule of a contiguous region in genomic DNA, wherein said contiguous region in genomic DNA is represented by a plurality of nucleic acid molecules, and wherein each of said first plurality of segments is appended to a first multiplex identifier (MID) sequence; and b) a second plurality of different segments of a second nucleic acid molecule of said contiguous region, wherein each of said second plurality of segments is appended to a second MID sequence and said first MID sequence and said second MID sequence are different, wherein, in said composition, all of the segments of each of the nucleic acid molecules of the contiguous region comprise a unique MID sequence that uniquely identifies each of the nucleic acid molecules, wherein said genomic DNA is genomic DNA from a virus or a bacterium.

One having ordinary skill in the art would recognize that genomic DNA from a bacterium amounts of a genome from a single isolated cell. One having ordinary skill in

the art would also recognize that providing a genome from a second isolated cell would be a duplication of the steps of '274 patent because instant claims as recited merely require analysis of the first and second appended segments and not linking the sequence to the first and the second isolated single cell.

However, claims of '274 patent do not disclose nucleic acid segments are from first and second cell and not using the composition for performing the method. However, providing genomic DNA from two different bacteria was known in the art at the time the claimed invention was made as taught by Drmanac, who teaches a method for analyzing mixtures of bacterial genomes, aliquots may be made so that each aliquot contains one cell type, or a maximum of one cell (paragraph 0043) and further teaches method for tracking the nucleic acid using oligonucleotide tag (i.e., identifier) for tracking each polynucleotide (paragraph 0105). Drmanac also teaches analyzing single cell nucleic acid DNA allows for more accurate pathogen diagnostic for health or biodefense applications (paragraph 0043), thus providing motivation to one of ordinary skill in the art to include the step of analyzing nucleic acid from a plurality of individual single cell of Drmanac in the method of '274 patent. One having ordinary skill in the art would recognize that the composition of claims 1-11 of '274 patent used to carry out the method because claimed method merely requires providing the plurality of segments of nucleic acid molecules, which is also taught by Drmanac.

The routine, well established and conventional method of identifying the polynucleotides in each sample from the pooled samples using unique tags for each

sample nucleic acids as applied for the rejection of claims 40 and 53 are further evidenced by Gillevet (Fig. 1 and pg. 17 and paragraph 3).

One having ordinary skill in the art would also recognize that while combining the composition of '274 patent with the step of Drmanac some routine optimization is necessary which is within the skills of one having ordinary skill in the art and motivation to include the missing step comes from Drmanac.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the step of analyzing nucleic acid from a plurality of individual single cell of Drmanac in the method of '274 patent with a reasonable expectation of success with the expected benefit of more accurate pathogen diagnosis. An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including the two single cell analysis which is routinely done in the art and therefore the steps of instant claims 40 and 53 are not patentably distinct from claims of '274 patent in view of Drmanac.

With regard to instant dependent claims 41-52, 54 and 55, claims of '274 patent in view of Drmanac disclose the steps and therefore are not patentably distinct.

22. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-13 of U.S. Patent No. 9,410,149 in view of Drmanac (US 2007 /0072208 published Mar. 29, 2007) for the following reasons.

The subject matter of claims 1-13 of '149 patent is similar to claims 1-11 of '756 patent. Therefore it is maintained that steps of instant claims 40-55 are not significantly different from claims 1-13 of '149 patent in view of Drmanac.

23. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-19 of U.S. Patent No. 9,410,150 in view of Drmanac (US 2007 /0072208 published Mar. 29, 2007) for the following reasons.

The subject matter of claims 1-19 of '150 patent is similar to claims 1-11 of '274 patent. Therefore it is maintained that steps of instant claims 40-55 are not significantly different from claims 1-19 of '150 patent in view of Drmanac.

Conclusion

24. No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to NARAYAN BHAT whose telephone number is (571)272-5540. The examiner can normally be reached on 8.30 am to 7 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave Nguyen can be reached on (571)-272-0731. 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.

/NARAYAN BHAT/
Primary Examiner, Art Unit 1634

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	B	US-				
	C	US-				
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Format: Summary Sort by: Publication Date

Search results

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(71) Applicant *(for all designated States except US)*: **BIOSPHEREX LLC** [US/US]; Suite 200, 15 Wiltshire Court East, Potomac Falls, Virginia 20165-5677 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **GILLEVET, Patrick, M.** [CA/US]; 11627 Ayreshire Rd. Oakton, Virginia 22124 (US).

(74) Agents: **MILLSTEIN, Larry, S.** et al.; Holland & Knight LLP, Suite 700, 1600 Tysons Boulevard, Mclean, VA 22102-4867 (US).

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(54) Title: MULTITAG SEQUENCING AND ECOGENOMICS ANALYSIS

(57) Abstract: Embodiments of the invention herein described relate to multiplex polynucleotide sequence analysis without the use of size separation methods or blotting. In certain particulars the invention relates to multiplex sequencing using massively parallel sequencing methods, such as pyrosequencing methods and sequencing by synthesis. The invention provides increased throughput and the ability to analyze greater numbers of samples simultaneously or serially on presently available systems, as well as others yet to be developed. In certain of its embodiments the invention relates to the analysis of complex microbial communities, particularly to in-depth analysis thereof in large numbers of samples.

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MULTITAG SEQUENCING AND ECOGENOMICS ANALYSIS

Related Applications

This application is a continuation in part and claims full benefit of priority of US provisional application number 60/858,948, filed on 15 November 2006 by Patrick Gillevet for Multitag Sequencing and Ecogenomics Analysis, which is incorporated herein by reference in its entirety.

Field of the Invention

The invention relates to the determination of polynucleotide sequences. It also relates to determining sequences in multiple samples, in some particulars in multiple environmental samples and in multiple clinical samples.

Statement of Government Rights

Work described herein was done partly with Government support under Grant No. 1R43DK074275-01A2 awarded by the U.S. National Institute of Diabetes and Digestive and Kidney Diseases, and the US Government therefore may have certain rights in the invention.

Background

Sequence determination technologies for proteins, RNAs and DNAs, have been pivotal in the development of modern molecular biology. During the past fifteen years, DNA sequencing in particular has been the core technology in an on-going revolution in the scope and the depth of understanding of genomic organization and function. The on-going development of sequencing technology is, perhaps, best symbolized by the determination of the complete sequence of a human genome.

The human genome sequencing project served a number of purposes. It served as a platform for programmatic development of improved sequencing technologies and of genome sequencing efforts. It also served to provide a framework for the production and distribution of sequencing information from increasingly large scale sequencing projects. These projects provided complete genome sequences for a succession of model organisms of increasingly large genetic complements. These accomplishments, culminating in the completion of a human genome sequence, highlight the very considerable power and throughput of contemporary sequencing technology.

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At the same time, however, they highlight the limitations of current technology and the need for considerable improvements in speed, accuracy, and cost before sequencing can be fully exploited in research and medicine. Among the areas that can be seen most readily to require advances in sequencing technology are clinical sequencing applications that require whole genome information, environmental applications involving multiple organisms in mixtures, and applications that require processing of many samples. These are, of course, just a few among a great many areas that either require or will benefit greatly from more capable and less expensive sequencing methods.

To date, virtually all sequencing has been done by Sanger chain elongation methods. All Sanger methods require separating the elongation products with single base resolution. Currently, while PAGE still is used for this purpose in some commercial sequencers, capillary electrophoresis is the method of choice for high throughput DNA sequencers. Both gel-based and capillary-based separation methods are time consuming, costly, and limit throughput. Chip based methods, such as Affymetrix GeneChips and HySeq's sequencing by hybridization methods, require chips that can be produced only by capital intensive and complex manufacturing processes. These limitations pose obstacles to the utilization of sequencing for many purposes, such as those described above. Partly to overcome the limitations imposed by the necessity for powerful separation techniques in chain termination sequencing methods and the manufacturing requirements of chip-based methods, a number of technologies are currently being developed that do not require the separation of elongation products with integer resolution and do not require chips.

A lead technology of this type is a bead, emulsion amplification, and pyrosequencing-based method developed by 454 Life Sciences. (See Margulies, et al. (2005) Nature 437: 376, which is incorporated herein by reference in its entirety, particularly as to the afore-mentioned methods. The method utilizes a series of steps to deposit single, amplified DNA molecules in individual wells of a plate containing several million picoliter wells. The steps ensure that each well of the plate either contains no DNA or the amplified DNA from a single original molecule. Pyrosequencing is carried out in the wells by elongation of a primer template in much the same way as Sanger sequencing. Pyrosequencing does not involve chain termination and does not require separation of elongation products. Instead sequencing proceeds stepwise by single base addition cycles. In each cycle one of the four bases – A, T, G, or C – is included in the elongation reaction. The other three bases are omitted. A base is added to the growing chain if it is complementary to the next position on the template. Light

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is produced whenever a base is incorporated into the growing complimentary sequence. By interrogating with each of A, C, G, or T in succession, the identity of the base at each position can be determined. Sequencing reactions are carried out in many wells simultaneously. Signals are collected from all the wells at once using an imaging detector. Thus, a multitude of sequences can be determined at the same time

In principle, each well containing a DNA will emit a signal for only one of the four bases for each position. In practice, runs of the same base at two or more positions in succession lead to the emission of proportionally stronger signals for the first position in the run. Consequently, reading out the sequence from a given well is a bit more complicated then simply noting, for each position, which of the four bases is added. Nevertheless, because signals are proportional to the number of incorporations, sequences can be accurately reconstructed from the signal strength for most runs.

The technology has been shown to read accurately an average of about 250 or so bases per well with acceptable accuracy. A device offered by 454 Life Sciences currently uses a 6.4 cm^2 picoliter well "plate" containing 1,600,000 picoliter sized wells for sequencing about 400,000 different templates. The throughput for a single run using this plate currently is about 100 million bases in four hours. Even though this is a first generation device, its throughput is nearly 100 times better than standard Sanger sequencing devices.

Numerous other methods are being developed for ultra high throughput sequencing by other institutions and companies. Sequencing by synthesis methods that rely on target amplification are being developed and/or commercialized by George Church at Harvard University, by Solexa, and by others. Ligation sequencing methods have been developed and/or are being commercialized by Applied Biosystems and Solexa, among others. Array and hybridization sequencing methods are commercially available and/or are being developed by Affymetrix, Hyseq, Biotrove, Nimblegen, Illumina, and others. Methods of sequencing single molecules are being pursued by Helicos based on sequencing by synthesis and U.S. Genomics (among others) based on poration.

These methods represent a considerable improvement in throughput over past methods, in some regards. And they promise considerable improvement in economy as well. However, currently they are expensive to implement and use, they are limited to relatively short reads and, although massively parallel, they have limitations that must be overcome to realize their full potential.

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One particular disadvantage of these methods, for example, is that samples must be processed serially, reducing throughput and increasing cost. This is a particularly great disadvantage when large numbers of samples are being processed, such as may be the case in clinical studies and environmental sampling, to name just two applications.

The incorporation of indexing sequences by ligation to random shotgun libraries has been disclosed in US patent numbers: 7264929, 7244559, and 7211390, but the direct ligation methods therein disclosed distort the distribution of the components within the samples (as illustrated in Figure 4 herein) and therefore are inappropriate for enumerating components within each sample.

Accordingly, there is a need to improve sample throughput, to lower the costs of sequencing polynucleotides from many samples at a time, and to accurately enumerate the components of samples analyzed by high throughput, parallelized and multiplex techniques. .

Summary

It is therefore an object of the present invention to provide sequencing methods with improved sample throughput. The following paragraphs describe a few illustrative embodiments of the invention that exemplify some of its aspects and features. They are not exhaustive in illustrating its many aspects and embodiments, and thus are not in any way limitative of the invention. Many other aspects, features, and embodiments of the invention are described herein. Many other aspects and embodiments will be readily apparent to those skilled in the art upon reading the application and giving it due consideration in the full light of the prior art and knowledge in the field.

Embodiments provide multiplex methods for the quantitative determination of polynucleotides in two or more samples, comprising:

hybridizing a first primer to polynucleotides in a first sample, said first primer comprising a first tag sequence and a first probe sequence specific for a first target sequence, wherein said first target sequence is 3' to a variable genetic region;

elongating primer templates formed thereby to form a first population of tagged polynucleotides comprising: said first primer including said first tag sequence; and sequences of said variable genetic region;

hybridizing a second primer to polynucleotides in a second sample, said second primer comprising a second tag sequence and a second probe sequence specific for a second target sequence, wherein said second target sequence is 3' to the same variable genetic region as said

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first target sequence, wherein further said second probe sequence may be the same as or different from said first probe sequence;

elongating primer templates formed thereby to form a second population of tagged polynucleotides comprising: said second primer including said second tag sequence; and sequences of said variable genetic region;

mixing said first and second populations together;

determining sequences of polynucleotides comprising tag sequences and the sequences of the variable genetic element in said mixture;

from the tag sequences comprised in the polynucleotide sequences thus determined identifying the sample in which polynucleotide sequences occurred;

from the sequences of the variable genetic region comprised in the polynucleotide sequences thus determined identifying particular variants of said variable genetic element;

from this information determining the number of time one or more given variants occur in each sample, and

from the number for each variant in the polynucleotides thus determined, quantifying said polynucleotides in said samples;

wherein said sequences are determined without Southern blot transfer and/or without size-separating primer extension products and/or without electrophoresis.

Embodiments provide multiplex methods for the quantitative determination of polynucleotides in two or more samples, comprising:

hybridizing a first primer pair to polynucleotides in a first sample, the first primer of said first primer pair comprising a first tag sequence and a first probe sequences specific for a first target sequence and the second primer of said first primer pair comprising a second tag sequence and a second probe sequence specific for a second target sequence, wherein the first and the second probe sequences flank and hybridize to opposite strands of a variable genetic region;

elongating primer templates formed thereby to from a first population of tagged polynucleotides, each of said polynucleotides comprising: (a) the sequence of said first primer of said first primer pair, a sequence of said variable genetic region, and a sequence complementary to the sequence of said second primer of said first primer pair or (b) a sequence complementary to the sequence of said first primer of said first primer pair, a sequence of said variable genetic region and the sequence of said second primer of said first primer pair;

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hybridizing a second primer pair to polynucleotides in a second sample, the first primer of said second primer pair comprising a third tag sequence and said first probe sequences specific for said first target sequence and the second primer of said second primer pair comprising a fourth tag sequence and said second probe sequence specific for said second target sequence;

elongating primer templates formed thereby to from a second population of tagged polynucleotides, each of said polynucleotides comprising: (a) the sequence of said first primer of said second primer pair, a sequence of said variable genetic region, and a sequence complementary to the sequence of said second primer of said second primer pair or (b) a sequence complementary to the sequence of said first primer of said second primer pair, a sequence of said variable genetic region and the sequence of said second primer of said second primer pair;

mixing said first and second populations together;

determining sequences of polynucleotides in said mixture, comprising the tag sequences and the variable genetic element;

from the tag sequences comprised in the polynucleotide sequences thus determined identifying the sample in which polynucleotide sequences occurred;

from the sequences of the variable genetic region comprised in the polynucleotide sequences thus determined identifying particular variants of said variable genetic element;

from this information determining the number of times given variants occur in each sample, and

from the number for each variant in the polynucleotides thus determined, quantifying said polynucleotides in said samples.

wherein said sequences are determined without Southern blot transfer and/or without size-separating primer extension products and/or without electrophoresis.

Embodiments provide methods in accordance with any of the foregoing or the following wherein given polynucleotide sequences in a sample is quantified by a method comprising normalizing the number occurrences determined for the given sequence. In embodiments the number of occurrences is normalized by dividing the number of occurrences determined for the given polynucleotide sequence by the total number of occurrences of polynucleotide sequences in the sample. In embodiments the given polynucleotide sequences is that of a given variant of a variable genetic region and, in embodiments, the quantity of the given variant in the sample is normalized by dividing the number of occurrences of that

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variant by the total number of occurrences of all variants of the variable genetic region in the sample.

Embodiments provide a multiplex method for determining polynucleotide sequences in two or more samples, comprising: attaching a first tag sequence to one or more polynucleotides of a first sample; attaching a second tag sequence different from said first tag sequence to one or more polynucleotides of a second sample; mixing the tagged polynucleotides of said first and second samples together; determining sequences of said polynucleotides comprising said first and said second tags; and identifying said first and second tags in said sequences; thereby identifying sequences of said polynucleotides of said first sample and second samples, wherein said sequences are determined without Southern blot transfer and/or without size-separating primer extension products and/or without electrophoresis.

Embodiments provide a multiplex method for determining polynucleotide sequences in two or more samples comprising:

attaching a first tag sequence, t_1, to $P_{1\text{-}1}$ through $P_{1\text{-}n1}$ polynucleotides in a first sample, thereby to provide a first plurality of polynucleotides tagged with said first tag, $t_1P_{1\text{-}1}$ through $t_1P_{1\text{-}n1}$;

attaching a second tag sequence, t_2, to $P_{2\text{-}1}$ through $P_{2\text{-}n2}$ polynucleotides in a second sample, thereby to provide a second plurality of polynucleotides tagged with said second tag, $t_2P_{2\text{-}1}$ through $t_2P_{2\text{-}n2}$;

mixing together said polynucleotides tagged with said first and said second tags;

determining sequences of polynucleotides comprising said tags in said mixture;

identifying said first and second tags in said sequences and;

by said first tag identifying polynucleotide sequences of said first sample and by said second tag identifying polynucleotide sequences of said second sample;

wherein said sequences are determined without Southern blot transfer and/or without size-separating primer extension products and/or without electrophoresis.

Embodiments provide a method according to any of the foregoing or the following, wherein the number of said polynucleotides in said first sample, n_1, is any of 2, 5, 10, 25, 50, 100, 150, 200, 250, 500, 1,000, 1,500, 2,000, 2,500, 5,000, 7,500, 10,000, 12,500, 15,000, 17,500, 20,000, 25,000, 30,000, 35,000, 40,000, 50,000, 75,000, 100,000, 150,000, 200,000, 250,000, 500,000, 1,000,000 or more, and the number of said polynucleotides in said second sample, n_2, is any of 2, 5, 10, 25, 50, 100, 150, 200, 250, 500, 1,000, 1,500, 2,000, 2,500,

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5,000, 7,500, 10,000, 12,500, 15,000, 17,500, 20,000, 25,000, 30,000, 35,000, 40,000, 50,000, 75,000, 100,000, 150,000, 200,000, 250,000, 500,000, 1,000,000 or more.

Embodiments provide a method according to any of the foregoing or the following, wherein the number of said samples and of said different tags therefor is 5, 10, 15, 20, 25, 50, 75, 100, 150, 200, 250, 500, 1,000, 2,500, 5,000, 10,000 or more.

Embodiments provide a method according to any of the foregoing or the following, wherein the tags are nucleotide sequences that are 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36 nucleotides long or any combination thereof.

Embodiments provide a method according to any of the foregoing or the following, wherein the tags are incorporated into said polynucleotides by a step of ligation, provided that the step of ligation does not result in biasing.

Embodiments provide a method according to any of the foregoing or the following, wherein the tags are incorporated into said polynucleotides by a step of ligation and/or by a step of amplification.

Embodiments provide a method according to any of the foregoing or the following, wherein said tags are comprised in primers for amplification and are incorporated into said polynucleotides by amplification using said primers.

Embodiments provide a method according to any of the foregoing or the following, wherein said tags are incorporated into said polynucleotides by a process comprising a step of cloning into a vector.

Embodiments provide a method according to any of the foregoing or the following, wherein the tags are comprised in adapters for amplification and said adapters are ligated to polynucleotides in said samples. Embodiments provide a method in this regard, wherein further, said polynucleotides ligated thereby to said tags are amplified via said adapters. Embodiments provide a method in this regard, wherein further, said adapters comprise a moiety for immobilization. In embodiments said moiety is a ligand; in embodiments it is biotin. Embodiments provide a method in this regard, wherein further, said tags are comprised on adapters for bead emulsion amplification. In embodiments the adapters are suitable for use in a sequencing system of 454 Life Sciences or other sequencing system in which bead emulsion amplification is carried out.

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Embodiments provide a method according to any of the foregoing or the following, wherein the primer for amplification comprises a sequence for PCR amplification, linear amplification, transcriptional amplification, rolling circle replication, or QB replication.

Embodiments provide a method according to any of the foregoing or the following, wherein the primer for amplification comprises a sequence for PCR amplification.

Embodiments provide a method according to any of the foregoing or the following, wherein each of said polynucleotides is disposed individually on a bead isolated from other polynucleotides.

Embodiments provide a method according to any of the foregoing or the following, wherein each of said polynucleotides is disposed individually on a bead isolated from other said polynucleotides, is amplified while disposed therein, and the amplification products thereof also are disposed on said bead.

Embodiments provide a method according to any of the foregoing or the following, wherein each of said polynucleotides is disposed individually on a bead isolated from other said polynucleotides, is amplified while disposed therein, the amplification products thereof also are disposed on said bead, and each said bead is disposed individually in a well isolated from other said beads.

Embodiments provide a method according to any of the foregoing or the following, wherein the sequences are determined by pyrosequencing.

Embodiments provide a method according to any of the foregoing or the following, wherein said samples are biological samples, each comprising one or more species.

Embodiments provide a method according to any of the foregoing or the following, wherein at least one sequence of said polynucleotides is specific to a particular organism.

Embodiments provide a method according to any of the foregoing or the following, wherein said sequences comprise a variable 16S rRNA sequence.

Embodiments provide a method according to any of the foregoing or the following, wherein said sequences comprise a variable 18S rRNA sequence, a variable rRNA ITS sequence, a mitochondrial sequence, a microsatellite sequence, a metabolic enzyme sequence, and/or a genetic disease sequence.

Embodiments provide a method according to any of the foregoing or the following, wherein the samples are microbial community samples.

Embodiments provide a method according to any of the foregoing or the following, wherein the samples are microbial community samples for clinical analysis of a patient.

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Embodiments provide a method according to any of the foregoing or the following, wherein the samples are microbial community environmental samples.

Embodiments provide a method according to any of the foregoing or the following, wherein the samples are microbial community soil samples.

Embodiments provide a method according to any of the foregoing or the following, wherein the samples are microbial community water samples.

Embodiments provide a method according to any of the foregoing or the following, wherein the samples are samples for SNP analysis.

Embodiments provide a method according to any of the foregoing or the following, wherein the samples are samples for genotyping.

Embodiments provide a multiplex method according to any of the foregoing or the following for determining polynucleotide sequences of two or more samples, comprising,

amplifying polynucleotides of a first sample to produce first amplified polynucleotides comprising a first tag sequence;

separately amplifying polynucleotides of a second sample to produce second amplified polynucleotides comprising a second tag sequence different from said first tag sequence;

wherein the amplification products arising from different individual polynucleotides are spatially separated from one another;

mixing together amplicons of said first and second samples;

distributing the amplicons in the mixture into spatially distinct locations; sequencing the amplicons thus distributed using one or more primers that hybridize 5' to said tag sequences;

identifying said tag sequences in the sequences of polynucleotides thus determined; and

identifying by said tags polynucleotides of said first sample and polynucleotides of said second sample.

Embodiments provide a method according to any of the foregoing or the following, comprising,

(a) for each sample separately: isolating polynucleotides to be sequenced, ligating said polynucleotides to a common adaptor comprising a tag sequence, and capturing individual ligated polynucleotides onto individual beads under conditions that provide predominately for the immobilization of 0 or 1 molecule per bead;

(b) thereafter mixing together said beads comprising said polynucleotides.

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Embodiments provide a method according to any of the foregoing or the following, further comprising, amplifying bead-immobilized polynucleotides in droplets of an emulsion thereby to clonally amplify said individual polynucleotides on said beads, wherein amplification comprises amplification of said tag sequence.

Embodiments provide a method according to any of the foregoing or the following, further comprising, distributing individual droplets containing said amplified polynucleotides into wells under conditions that provide predominantly for 0 or 1 droplet per well, determining in individual wells the sequences of polynucleotides comprising said tag sequences, and by said tag sequences identifying polynucleotides of said first and said second samples.

In embodiments the invention provides methods in accordance with any of the foregoing or the following, for any one or more of detecting, monitoring, profiling, prognosticating, and/or diagnosing a disorder, disease, or the like.

In embodiments the invention provides methods in accordance with any of the foregoing or the following, for analyzing the composition, diversity, stability, dynamics, and/or changes in agricultural, food, biosecurity, veterinary, clinical, ecological, zoological, oceanological, and/or any other sample comprising one or more polynucleotides.

Embodiments provide kits comprising a plurality of two or more primers, each primer in said plurality comprising a tag sequence and a probe sequence specific to a target sequence, wherein:

(A) in each of said primers the probe sequence is 3' to the tag sequence, but not necessarily adjacent thereto;

(B) in each of said primers: the tag sequence is different from the tag sequence of the other in the plurality; the tag sequence is not the complementary sequence to any other tag sequence in the plurality; the tag sequence does not contain any homodinucleotide sequences; the junction sequences between the tag sequence and the adjacent parts of the primer, if any, is not a homodinucleotide sequence;

(C) in each of said primers the probe sequence is complementary to the target sequence and the target sequence is located 3' to a variable genetic region, and

(D) each of said primers is disposed separately from the others in containers in said kit.

Embodiments provide kits in accordance with any of the foregoing or the following, wherein each of said primers further comprises a priming sequence 5' to the tag sequence but not necessarily adjacent thereto, and the priming sequence is the same in all of said primers,

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said kit further comprising a primer complimentary to and effective for polymerization from said priming sequence.

Embodiments provides kits comprising a plurality of two or more primers pairs, each primer in said plurality comprising a tag sequence and a probe sequence specific to a target sequence, wherein:

(A) in each of said primer the probe sequence is 3' to the tag sequence, but not necessarily adjacent thereto;

(B) in each of said primers: the tag sequence is different from the tag sequence of the other in the plurality; the tag sequence is not the complementary sequence to any other tag sequence in the plurality; the tag sequence does not contain any homodinucleotide sequences; the junction sequences between the tag sequence and the adjacent parts of the primer, if any, is not a homodinucleotide sequence;

(C) in each of said primers the probe sequence is complementary to the target sequence,

(D) in each primer pair the probe sequences are specific to target sequences that flank a variable genetic region;

(E) each of said primers is disposed separately from the others in said kit.

Embodiments provides kits in accordance with any of the foregoing or the following, wherein, the primers further comprise a priming sequence 5' to the tag sequence but not necessarily adjacent thereto, the priming sequence either is the same in all the primers, or one member of each pair has the same first priming sequence and the second member of each pair has the same second priming sequence, said kit further comprising disposed separately from one another in one or more containers one or more primers complementary to and effective for elongation from said priming

Embodiments provide a kit useful in methods according to any of the foregoing or the following, comprising a set of primers and/or adapters, wherein each primer and/or adapter in said set comprises a tag sequence and a primer sequence. In embodiments the primers and/or adapters further comprise a moiety for immobilization. In embodiments the primers and/or adapters comprise biotin. In embodiments the primers and/or adapters in the set comprise all tag sequences defined by 2, 3, 4, 5, 6, 7, or 8 base polynucleotide sequences, wherein each of said primers and/or adapters are disposed in containers separate from one another. In embodiments there are 1-5, 3-10, 5-15, 10-25, 20-50, 25-75, 50-100, 50-150, 100-200, 150-500, 250-750, 100-1000, or more different tag sequences disposed separately from one

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another, so as to be useful for uniquely tagging said number of different samples. In embodiments the primers and/or adapters are suitable for use as 454 Life Sciences amplification adapters and/or primers. In embodiments the primers and/or adapters further comprise any one or more of a primer sequence for any one or more of a 16S rRNA sequence, an 18S rRNA sequence, an ITS sequence, a mitochondrial sequence, a microsatellite sequence, a metabolic enzyme sequence, a genetic disease sequence, and/or any other sequence for amplification or analysis.

In embodiments the invention provides a kit, in accordance with any of the foregoing or the following, comprising a set of primers and/or adapters for use in a method according to any of the foregoing or the following, wherein each primer and/or adapter in said set comprises a tag sequence, the tag sequence of each of said primers and/or adapters is different from that of the other primers and/or adapters in said set, the primers and/or adapters further comprise a priming sequence that is the same in all of the primers and/or adapters in said set, the tag sequences are located 5' to the priming sequence and the different primers and/or adapters comprising each different tag sequence are disposed separately from one another. In embodiments the tags are any number of bases long. In embodiments the tags are 2, 3, 4, 5, 6, 8, 10, 12 bases long. In embodiments the tags are 4 bases long. In embodiments the priming sequence is specific to any target polynucleotide of interest. In embodiments the priming sequence is specific to a sequence in 16S rRNA. In embodiments the tags differ from each other by at least 2 bases. In embodiments the tags do not contain polynucleotide tracts within the tag. In embodiments the tags do not contain homo-polynucleotide tracts within or at the junction of the tag and PCR primer. In embodiments the tags do not contain polynucleotide tracts within or at the junction of the tag and emulsion PCR adapter. In embodiments, the tags are not reverse compliments of each other.

Brief Description of the Figures

Figure 1 is a schematic diagram showing a general embodiment of the invention. A plurality of samples (S_1, S_2, through S_j) is shown topmost in the Figure. Each sample is comprised of a plurality of polynucleotides (P_{1-1} to P_{1-n1} in S_1; P_{2-1} to P_{2-n2} in S2; through P_{j-1} to P_{j-nj}). The polynucleotides in each sample are labeled separately with a tag polynucleotide sequence, all the polynucleotides in a given sample being tagged (in this illustration) with a single tag sequence, designated in the figure as T_1 for S_1, T_2 for S_2, through T_j for S_j. The individual tagged polynucleotides are denoted accordingly. The tagged polynucleotides in

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each sample are designated collectively, for each sample, T_1S_1, T_2S_2, through T_jS_i. The tagged polynucleotides from the samples are mixed together to form a mixture, designated M_1. The mixture is sequenced, typically by a massively parallel sequencing method. The tag sequences are identified in the data thus obtained. The sequences are grouped by tag. The sequences from the individual samples are thereby identified.

Figure 2A is a diagram depicting step I in the multitag sequencing of microbial community samples using a tagged 16S forward and reverse primer-linker pairs for PCR amplification. (a) represents the Forward 16S rRNA primer with Tag *I* and Emulsion PCR Linker, (b) represents the 16S rRNA sequence, (c) represents the Reverse 16S rRNA primer with Tag *j* and Emulsion PCR Linker, (d) represents the Amplified 16S rRNA sequence with Forward and Reverse Tags *ij*, (e) represents the Emulsion PCR Bead, (f) represents the pyrosequencing read, (g) represents the well in picoliter plate, (h) represents a Unique tag, (i) represents Amplified Community 1, (j) represents Amplified Community 2, and (k) represents Amplified Community *n*. Step 1 involves the amplification of the microbial community from each sample using uniquely tagged universal primers-linkers. In step 1, different samples are amplified separately, using 16S rRNA specific adapter-tag-primers with a different tag for each sample.

Figure 2B is a diagram depicting the Emulsion PCR reaction beads randomly arrayed into picoliter plate. In step 2 in the process, the PCR products from all the samples are mixed, immobilized on beads, distributed into wells of the picoliter plate, and emulsion PCR amplified.

Figure 2C is a diagram depicting the pyrosequencing process from each outside adapter in each well of the picoliter plate. Each reaction reads sequence from the adapter, through the unique tags and the associated sequence of the tagged sample

Figure 2D is a diagram depicting the algorithmic sorting of the Pyrosequencing reads using the individual tag sequence and a portion of the primer sequence. (l) represents the sequence reads from sample 1, (m) represents the sequence reads from sample 2, and (n) represents the sequence reads from sample n.

Figure 2E is a diagram depicting the identification of microbial taxa by comparing the sequence reads for each sample against the 16S rRNA sequence database and then normalize abundance in each taxa with respect to the total reads in that particular sample. (o) represents the normalized species histogram derived the pyrosequencing reads obtained from sample 1, (p) represents the normalized species histogram derived the pyrosequencing reads obtained

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from sample 2, (q) represents the normalized species histogram derived the pyrosequencing reads obtained from sample n,

Figure 3 is the species distribution in (A) Controls, (B) Crohns, and (C) Ulcerative colitis samples determined by the 454 Life Science pyrosequencing process. Each bar in the histogram is the average normalized abundance of that taxa in each disease state. Each sample was run in a separate well on the picoliter plate using the 454 16 well mask.

Figure 4 is an example of the distortion of the components of a complex mixture caused by ligating the Emulsion PCR adapters onto PCR amplicons. Figure 4A shows the size distribution of PCR amplicons in sample 309 before ligation and Figure 4B shows the size distribution of sample 309 after ligation.

Figure 5 is an example of the normalized taxa abundances in duplicate samples determined by Multitag pyrosequencing after direct ligation of the emulsion PCR adapters.

Figure 6 shows all possible hexameric polynucleotide tags within which there are no dinucleotide repeats and no tag is the reverse complement of any other tag.

Figure 7 shows 96 tagged adaptor primers in which there are no dinucleotide repeats in the tags, no dinucleotide repeats at the junction of the tags and the tags are not reverse complements of one another. In each case 5 bases of the primer also can be used to identify samples. 7A and 7B show the forward primers. 7C and 7D show the reverse primers.

Glossary

The meanings ascribed to various terms and phrases as used herein are illustratively explained below.

"A" or "an" means one or more; at least one.

"About" as used herein means roughly, approximately. Should a precise numerical definition be required, "about" means +/- 25%.

"Adapter" means a polynucleotide sequence used to either attach single polynucleotide fragments to beads and/or to prime the emulsion PCR reaction and/or as a template to prime pyrosequencing reactions.

"ALH" is used herein to mean amplicon length heterogeneity.

"Amplicon" is used herein to refer to the products of an amplification reaction.

"Clonally amplified" is used herein generally to mean amplification of a single starting molecule. Typically it also refers to the clustering together of the amplification products, isolated from other amplification templates or products.

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"dsDNA" means double stranded DNA.

Dysbiosis means a shift in a the species and abundance of species in a microbial community.

"Flanking" generally is used to mean on each side, such as on the 5' and the 3' side of a region of a polynucleotide – with reference to the 5' and the 3' ends of one or the other stand of a double stranded polynucleotide. Forward and reverse primers for amplifying a region of a polynucleotide by PCR, for instance, flank the region to be amplified.

"Microbial community sample" is used herein to refer to a sample, generally of a biological nature, containing two or more different microbes. Microbial community samples include, for instance, environmental samples, as well as biological samples, such as samples for clinical analysis. The term applies as well to preparations, such as DNA preparations, derived from such samples.

"Multiplex sequencing" herein refers to sequencing two or more types or samples of polynucleotides in a single reaction or in a single reaction vessel.

"PCO" means principal coordinates analysis.

"PCA" means principal component analysis.

"Picotiter plate" means a plate having a large number of wells that hold a relatively small volume, typically more wells than a 96-well microtiter plate, and smaller volumes than those of a typical 96-well microtiter plate well.

"Primer" means a polynucleotide sequence that is used to amplify PCR products and/or to prime sequencing reactions.

"ssDNA" means single stranded DNA.

"Tag," "Tag sequence," etc. means typically a heterologous sequence, such as a polynucleotide sequence that identifies another sequence with which it is associated as being of a given type or belonging to a given group.

"Variable genetic region" as used herein means a genetic region that varies, such as between individuals of a species and between species. The phrase does not denote a specific length, but, rather is used to denote a region comprising a variation the exact length of which may vary and may differ in different contexts. As to a double stranded polynucleotide, the term includes one or the other and both stands of the region, and may be used to refer to one, the other, or to both strands, and it will generally be clear from the context which is meant. A specific example of a genetic region that varies between individuals, provided for illustration only, is a genetic region that contains an SNP (single nucleotide polymorphism) site. By

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variable genetic region in this regard is meant a region containing the SNP site. Different sequences of the SNP in this regard constitute the variants of the variable genetic region. A specific example of a variable genetic region that differs between species is the genes for 16S RNA which vary characteristically between microbes and can be used to identify microbes in mixed community samples as described in greater detail in some of the examples herein.

Description of the Invention

In certain aspects and embodiments the invention relates to multiplex sequencing analysis using tags. In various aspects and embodiments of the invention in this regard the invention provides methods for sequencing two or more samples simultaneously in a mixture with one another, wherein each sample is first linked to a sample-specific sequence tag, the tagged samples are mixed and sequenced, and the sequences from each sample then are identified by their respective sample-specific sequence tags.

Figure 1 provides a general depiction of various aspects and embodiments of the invention in this regard, and the figure is discussed by way of illustration below with reference to sequencing DNA from different samples. A plurality of samples (S_1, S_2, through S_j) is shown topmost in the Figure. Each sample is comprised of a plurality of polynucleotides ($P_{1\text{-}1}$ to $P_{1\text{-}n1}$ in S_1; $P_{2\text{-}1}$ to$P_{2\text{-}n2}$ in S2; through $P_{j\text{-}1}$ to $P_{j\text{-}nj}$). The polynucleotides in each sample are labeled separately with a tag polynucleotide sequence, all the polynucleotides in a given sample being tagged (in this illustration) with a single tag sequence, designated in the figure as T_1 for S_1, T_2 for S_2, through T_j for S_j. The individual tagged polynucleotides are denoted accordingly. The tagged polynucleotides in each sample are designated collectively, for each sample, T_1S_1, T_2S_2 through T_jS_j. The tagged polynucleotides from the samples are mixed together to form a mixture, designated M_1. The mixture is sequenced typically by a parallel sequencing method. The tag sequences are identified in the data thus obtained. The sequences are grouped by tag. The sequences from the individual samples are thereby identified.

In embodiments tags are 3 to 30, 4 to 25, 4 to 20 base long sequences. In embodiments the tags are 2, 3 , 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36 nucleotides long or any combination thereof.

In embodiments there are 1 – 6, 6 – 12, 10 – 15, 10 – 20, 15 – 25, 20 – 40, 25 – 50, 25 – 75, 50 – 100, 50 – 150, 100 – 200, 100 – 250, 50 – 250, 100 – 500, 500 – 1,000, 100 –

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1,000, 500 – 5,000, 100 – 10,000, 1,000 – 25,000, 500 – 50,000, 100 – 100,000, 1 – 1,000,000 or more samples, tagged, respectively, with 1 – 6, 6 – 12, 10 – 15, 10 – 20, 15 – 25, 20 – 40, 25 – 50, 25 – 75, 50 – 100, 50 – 150, 100 – 200, 100 – 250, 50 – 250, 100 – 500, 500 – 1,000, 100 – 1,000, 500 – 5,000, 100 – 10,000, 1,000 – 25,000, 500 – 50,000, 100 – 100,000, 1 – 1,000,000 or more different tags.

In embodiments the sequences are determined without the use of gel electrophoresis. In embodiments the sequences are determined without the use of transfer of sequences from a gel onto a membrane or a filter for hybridization. In embodiments, sequences are determined by a parallel sequencing method. In embodiments the sequences are determined by pyrosequencing, sequencing by synthesis, hybridization sequencing, subtractive sequencing, pore sequencing or direct read sequencing.

In embodiments the tags are incorporated into polynucleotides in samples for sequencing by a step of ligation and/or by a step of amplification.

In embodiments the tags are comprised in primers for amplification.

In embodiments the tags are comprised in primers for PCR amplification, transcription amplification, rolling circle amplification, or amplification by Qβ replicase.

In embodiments the tags are comprised in emulsion PCR adapters and primers for amplification.

In embodiments the tags are incorporated by a step of cloning into a vector.

In embodiments the samples are microbial community samples. In embodiments the samples are clinical samples. In embodiments the samples are environmental samples. In embodiments the samples are samples for SNP analysis. In embodiments the samples are samples for genotyping. In embodiments the sequences are determined in one or more picotiter plates.

In embodiments the samples are fragmented genomic DNAs. In embodiments the samples are fragmented Bacterial genomic DNA, Archae genomic DNA, Fungal genomic DNA, Eukaryotic genomic DNA, chloroplast DNA, and/or mitochondrial DNA. In embodiments the samples are cDNAs. In embodiments the samples are Eukaryotic cDNA, Bacterial cDNA, Archae cDNA, and/or Fungal cDNA. In embodiments the tags are incorporated by a step of ligation and/or by a step of amplification.

In embodiments the samples are for any one or more of detecting, monitoring, profiling, prognosticating, and/or diagnosing a disorder, disease, or the like.

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In embodiments the samples are for analyzing the composition, diversity, stability, dynamics, and/or changes in agricultural, food, biosecurity, veterinary, clinical, ecological, zoological, oceanological, and/or any other sample comprising one or more polynucleotides.

In embodiments the sequences are determined in wells of a titer plate. In embodiments the sequences are determined in one or more picotiter plates having a mask. In embodiments the sequences are determined in one more picotiter plates having a mask, wherein the mask defines 2, 4, 8, 16, 32, 64 or more compartments.

By way of illustration to a 454 picotiter plate, in embodiments there are about 120,000 templates/plate and the read length averages about 250 bases per template. In embodiments relating thereto there are 10 tags of 4 bases per 1/16 plate, 160 tags total, an average of about 750 templates per tag (and per sample), and about 187,500 bases sequenced per tag (and per sample).

In embodiments there are about 260,000 templates/plate and the read length averages about 250 bases per template. In embodiments relating thereto, there are 12 tags of 4 bases per 1/8 plate, 96 samples total, an average of about 2,708 templates per tag (and per sample) and about 677,083 bases of sequence per tag (and per sample).

In embodiments there are about 400,000 templates/plate and the read length averages about 250 bases per template. In embodiments relating thereto, there are 96 tags of 6 bases for 96 samples per plate, about 4,166 templates per tag (and per sample) and about 1,041,666 bases of sequence per tag (and per sample).

In embodiments the tags are 10 base long sequences, there are 192 different tags, and the samples are analyzed in microtiter plate format.

In embodiments the invention provides algorithms for deconvolving, from a mixture of sequences from two or more samples, the sequences of the samples in the mixture by identifying sample-specific tags in the sequences, grouping the sequences by the tags thus identified, thereby grouping together the sequence from each of said samples, apart from one another.

In embodiments the invention provides algorithms for deconvolving, from a mixture of sequences from two or more samples, the sequences of the samples in the mixture by identifying sample-specific tags in sequences, as follows:

1. Read all sequence reads into an array;
2. Search the beginning of each sequence read and identify the tag;
3. Build an associative array linking tag with sequence read;

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4. Sort the keys for the associate array;
5. Associate each key with the corresponding sample;
6. Pool all sequence reads for each sample;
7. Analyze each sample separately.
8. Normalize the abundance of each component within each samples with respect to the total reads within that sample.

In embodiments the algorithm can be implemented in any programming language. In embodiments the algorithm is implemented in C, C++, JAVA, Fortran, or Basic. In embodiments the algorithm is implemented as a PERL script.

In embodiments the invention provides kits for multiplex sequencing as described herein, comprising a set of primers and/or adapters, wherein each primer and/or adapter in said set comprises a tag sequence, a primer sequence and/or an emulsion PCR adapter. In embodiments the primers and/or adapters further comprise a moiety for immobilization. In embodiments the primers and/or adapters comprise biotin. In embodiments the primers and/or adapters in the set comprise all tag sequences defined by 2, 3, 4, 5, 6, 7, or 8 base polynucleotide sequences, wherein said primers and/or adapters comprising different tag sequences are disposed in containers separate from one another. In embodiments there are 1-5, 3-10, 5-15, 10-25, 20-50, 25-75, 50-100, 50-150, 100-200, 150-500, 250-750, 100-1000, or more different tag sequences disposed separately from one another, so as to be useful for uniquely tagging said number of different samples. In embodiments the primers and/or adapters are suitable for use as 454 Life Sciences amplification adapters and/or primers. In embodiments the primers and/or adapters further comprise any one or more of a primer sequence for any one or more of a 16S rRNA sequence, an 18S rRNA sequence, an ITS sequence, a mitochondrial sequence, a microsatellite sequence, a metabolic enzyme sequence, a genetic disease sequence, and/or any other sequence for amplification or analysis.

Examples

The present invention is additionally described by way of the following illustrative, non-limiting examples.

EXAMPLE 1: Sequencing Using the 454 Pyrosequencing System

454 Life Sciences, a subsidiary of Roche Diagnostics, provides a device for pyrosequencing approximately 100,000,000 bases of about 400,000 different templates in a single run on a single picotiter plate. The company also provides masks that allows for the

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processing 2, 4, 8, or 16 different samples on one plate. At maximum capacity using the masked plate, the system provides about 1 million bases of sequence data on about 4,000 templates for each of 16 samples.

The general process of sequencing using the 454 system is generally as follows: isolate DNA; optionally fragment the DNA; optionally render the DNA double stranded; ligate the DNA to adaptors; separate the strands of the dsDNA, bind the ssDNA to beads under conditions that result in a preponderance of beads that have either no DNA molecule bound to them or a single molecule of DNA bound to them; capture the beads in individual droplets of an emulsion of a PCR reaction mix in oil; carry out a PCR reaction on the emulsion-encapsulated bead-DNAs (whereby amplification products are captured on the beads); distribute the amplification products into picoliter wells so that there is either no bead in a well or one bead; and carry out pyrosequencing on all the beads in all the wells in parallel.

EXAMPLE 2: Multiplex Pyrosequencing using 96 tagged adapter-PCR primers.

454 Life Sciences, a subsidiary of Roche Diagnostics, provides a device for pyrosequencing approximately 100,000,000 bases of sequence for about 400,000 different templates in a single run on a single picotiter plate. At maximum capacity using the plate, the system provides about 10 million bases of sequence data for each of about 4,000 templates for each of 96 multitagged samples. In this example the 96 tags are 6 bases in length and are used along with 6 bases of the forward or reverse primer to identify the reads that belong with each of the 96 individual samples (see Figure 2).

EXAMPLE 3: Multtag Pyrosequence Analysis of Microbial Community Samples

Various aspects and embodiments of the invention herein described are illustrated by way of the following general example relating to "ecogenomic" analysis of microbial diversity in biological samples.

The ability to quantify the number and kinds of microorganisms within a community is fundamental to the understanding of the structure and function of an ecosystem, as discussed in, for instance, Pace 1997 and Theron and Cloete 2000. Traditionally, the analysis of microbial communities has been conducted using microbiological techniques, but these techniques are limited. For instance they are not useful for the many organisms that cannot be cultivated (Ritchie, Schutter et al. 2000; Spring, Schulze et al. 2000). Even for those

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organisms that can be cultured, these techniques provide little information with which to identify individual microbes or characterize their physiological traits. (Morris, Bardin et al. 2002).

Recent advances in molecular techniques have overcome some of these disadvantages, and have enabled the identification of many more taxa in microbial communities than traditional microbial techniques. These advances have provided considerable insight into the expression of key functions in species in microbial communities. (Pace 1997; Suzuki 1998; Amann 2000; Frischer, Danforth et al. 2000; Ritchie, Schutter et al. 2000; Spring, Schulze et al. 2000). Among these molecular techniques are Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE), Temporal Temperature Gradient Gel Electrophoresis (TTGE), Terminal-Restriction Fragment Length Polymorphism (T-RFLP), Single Strand Conformation Polymorphism (SSCP), and Length Heterogeneity PCR (LH-PCR) (Frischer, Danforth et al. 2000; Theron and Cloete 2000; Mills, Fitzgerald et al. 2003; Seviour, Mino et al. 2003; Klaper and Thomas 2004).

Among these, LH-PCR is probably the best technique for fingerprinting. It is inexpensive, fast, and can be used routinely to screen several hundred samples a day. It is useful as a routine survey tool that can be used to monitor the dynamics of natural soil microbial communities, and to quickly identify samples of interest by PCO analysis. LH-PCR has been used to extensively assess natural variation in bacterial communities by profiling the amplified variable regions of 16S rRNA genes in mixed microbial population samples, using PAGE. (See Mills 2000; Litchfield and Gillevet 2002; Lydell, Dowell et al. 2004). The LH-PCR products of the individual species in the population give rise to distinct bands in the gels. The "peak area" of each band is proportional to the abundance of the species in the community. LH-PCR of 16S rRNA variable regions has been used quite successfully to estimate species diversity in bacterioplankton communities, in particular. (See Suzuki, Rappe et al. 1998; Ritchie, Schutter et al. 2000).

Community functionality cannot be determined directly from 16S rRNA clone data, however, it must be inferred from the data by phylogenetic analysis. Furthermore, LH-PCR and other fingerprinting technologies, while powerful tools for monitoring population dynamics, cannot identify individual species in a community. For this, fingerprinting investigations must be followed up by library construction, cloning, sequencing, and phylogenetic analysis. (Fitzgerald 1999; McCraig 1999; Spring, Schulze et al. 2000; Theron and Cloete 2000; Litchfield and Gillevet 2002; Bowman and McCuaig 2003; Kang and Mills

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2004; Eckburg, Bik et al. 2005). Identifying species of a fingerprinting study, thus, is a considerable undertaking that is inconvenient, time-consuming, expensive and subject to technical limitations.

Grouping samples can, to some extent, reduce the cost, time, and expense of such analyses. For instance, PCO analysis of LH-PCR data can be used to group samples with similar profiles for batch cloning and sequencing. Combining the samples this way reduces the time, expense, and work involved in analyzing the samples. Sequencing of at least 300 random clones is required to identify the bacterial components of the pooled sample down to 1% of the total bacterial populations in typical samples. This level of resolution is similar to that of ALH fingerprinting. Originally a novel approach, pooling similar samples prior to cloning and sequencing has proven to be robust and effective.

In classic community studies in the literature (Eckburg, Bik et al. 2005), environmental samples are assayed independently. Then the clone sequence data from specific classes/groups are statistically analyzed usually using some sort of averaging metric. Analyses of this type can be extremely costly, especially if the clone libraries are exhaustively analyzed, something that typically involves sequencing thousands of clones. Moreover, for the "averaging" process to be valid, as required for comparing the mixed populations, the samples must be pooled in equal proportions. While simple in principle, in reality, it is difficult to accomplish and, even if accomplished, impossible to verify. A new technique, based on pyrosequencing, offers advantages that overcome a variety of these drawbacks of the fingerprinting technologies mentioned above. The method is implemented on an instrument sold by 454 Life Sciences, Inc., a subsidiary of Curagen Sciences, Inc., using reagents provided by the same company. In addition, 454 Life Sciences provides a custom service for pyrosequencing.

In this technology, individual DNA molecules are amplified on beads by PCR in individual droplets in an oil-in-water emulsion. Beads then are deposited individually in wells of a picotiter plate. The sequences of all the DNAs in the wells are determined in parallel by pyrosequencing. (See Venter, Levy et al. 2003; Margulies, Egholm et al. 2005; Poinar, Schwarz et al. 2006). In a typical run, there are about 200,000 templates per plate, an average read length of about 100 bases from each template, and a single-plate run generates about 20 million bases of sequence in a single four hour run.

Although the technology greatly increases throughput over previous methods, it is expensive. In particular, the cost per plate is too high for it to be economically practical to

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carry out many analyses. To decrease cost, masks can be used that divide a plate into 16 independent sample zones, so that one plate can be used to process 16 different samples, either at the same time or independently. Each 1/16 zone provides about 1,000,000 bases of sequence data from about 10,000 different templates. While this reduces the cost per sample, the expenses associated with using this technology remain undesirably high.

Various aspects and embodiments of the present invention can be used to further reduce the cost per sample of this technology (as well as other techniques, as described elsewhere herein). The use of multitagging techniques (referred to as, among other things, "Multitag Process") to the genomic analysis of bacterial populations in according with certain aspects and embodiments of the invention, notably high coverage sequencing of bacterial communities, is referred to herein as "Multitag Ecogenomics" and also as "Multitag Ecogenomic Analysis."

(Several publications use the term "Multiplex Pyrosequencing" (Pourmand, Elahi et al. 2002) to refer to generating a composite signal from multiple targets that is read as a signature for a specific sample. The term is not used to refer to tag-based multiplexing in which sequences from different samples in a mixture are determined and then deconvolved from the mixed sequencing data using sample-specific tags incorporated during amplification reactions.)

As described below the Multitag Process in a relatively simple series of steps accomplishes everything that otherwise would require not only community fingerprinting analysis, but also all of the cloning and sequencing processes previously required for high coverage Ecogenomic Analysis using conventional techniques.

By way of illustration, the following example describes the use of Multitag Ecogenomic Analysis of variable regions of common genes using tagged universal primers for high coverage analysis of several microbial community samples all at the same time. The analysis is carried out much as described in general above, and further elaborated on in detail below.

Briefly, short tags are added to the 5' ends of the forward and reverse PCR primers normally used for community analysis. These tags can be placed between the Emulsion PCR adapters and the PCT primers (see Figure 2). A different tag is attached to the primers for each of the samples to be combined. For instance primers that span a variable region of 16S rRNA genes may be used for analysis of bacterial and archael communities. 16S rRNA-specific primers with 4 base tags are set out in the Table 1 below. Likewise primers that span

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a variable region of an ITS gene may be used for analysis of fungal communities. It will be appreciated that the choice of these specific primers is not exclusive, and that a wide variety of other primers suitable to other target regions for amplification may be employed in much the same manner as described herein for the 16S and ITS genes. Thus, any gene of interest can be used that provides conserved primer sites across a community, and sufficient variation in the region between the primers for the desired resolution of individual species. Thus, for example, genes specific to functional pathways such as anaerobic methane oxidation, or sulphur reduction can serve as targets for the amplification reaction, as well as 16S rRNA sequences.

Table 1

Name	Tag	Forward Shared Sequence
		AGCTAGAGTTTGATCMTGGCTCAG
L27FA	AGCT	AGCTAGAGTTTGATCMTGGCTCAG
L27FB	AGTC	AGTCAGAGTTTGATCMTGGCTCAG
L27FC	GATC	GATCAGAGTTTGATCMTGGCTCAG
L27FD	GACT	GACTAGAGTTTGATCMTGGCTCAG
L27FE	CTGC	CTGCAGAGTTTGATCMTGGCTCAG
L27FF	CTAG	CTAGAGAGTTTGATCMTGGCTCAG
L27FG	ATGC	ATGCAGAGTTTGATCMTGGCTCAG
L27FH	ATAG	ATAGAGAGTTTGATCMTGGCTCAG
L27FM	ATCT	ATCTAGAGTTTGATCMTGGCTCAG
L27FO	ATAT	ATATAGAGTTTGATCMTGGCTCAG
Name	**Tag**	**Reverse Shared Sequence**
		AGCTGCTGCCTCCCGTAGGAGT
355RA	AGCT	AGCTGCTGCCTCCCGTAGGAGT
355RB	AGTC	AGTCGCTGCCTCCCGTAGGAGT
355RC	GATC	GATCGCTGCCTCCCGTAGGAGT
355RD	GACT	GACTGCTGCCTCCCGTAGGAGT
355RE	CTGC	CTGCGCTGCCTCCCGTAGGAGT
355RF	CTAT	CTATGCTGCCTCCCGTAGGAGT
355RG	ATGC	ATGCGCTGCCTCCCGTAGGAGT
355RH	ATAT	ATATGCTGCCTCCCGTAGGAGT
355RM	ATCT	ATCTGCTGCCTCCCGTAGGAGT
355RO	ATAC	ATACGCTGCCTCCCGTAGGAGT

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Table 1 shows a 16S rRNA-specific primer with a variety of 4 base tag sequences attached. As described herein such primers are useful for amplifying 16S rRNAs in several samples that can then be sequenced together. The 16S rRNA in each sample is amplified using a different tag, but the same 16S primer sequence. The amplified rRNA sequences from the samples are combined and sequenced together. The rRNA sequences from the different samples then are identified and sorted out by their 4 base tag sequence plus the first 4 bases of each primer. It is to be appreciated that the sequences downstream of the shared 16S primer sequence will differ among the samples, as well as the tag sequence.

In each case, the samples are individually amplified. The resulting amplicons comprise the primer sequences including the tags. Since unique tags are used for each sample, the tags in the amplicons from each sample will be different. The amplified DNAs are then pooled and sequenced by pyrosequencing as described above. The sequence data from a run is analyzed, in part, by grouping together all the sequences having the same tag. In this way, the sequences from each sample are demultiplexed from the sequencing data obtained from the mixture.

The working of the invention in this regard is illustrated by the following simulation, carried out using conventionally obtained population data from cold seep samples. The algorithm for sequence analysis uses a PERL script to extract the first 100 bases of sequence. It then analyzes all the 100 bases sequences using a custom RDP PERL script. The script works as follows:

1. Read all sequence reads into an associate array (Hash 1);
2. Extract 100 base subsequences from the beginning of each sequence read;
3. Create an associate array (Hash 2) of the sequences;
4. Perform a Blast search of the RDP database with Hash 1;
5. Perform a Blast search of the RDP database with Hash 2;
6. Compare the identifications for the original sequence (Hash 1) and the subsequence (Hash 2);
7. Compile a list of similar identifications for Hash 1 and Hash 2;
8. Compile a list of different identifications for Hash 1 and Hash 2;
9. Calculate the percentage of similar identifications.

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As shown below, there is virtually no difference at the class level in the microbial diversity generated by the sequencing simulation and that derived directly from the 16S rRNA sequences in the data base.

Table 2

RDP Class	First 100mer	16S rRNA
ALPHA_SUBDIVISION	3.6%	3.6%
ANAEROBIC_HALOPHILES	3.6%	3.6%
BACILLUS-LACTOBACILLUS-STREPTOCOCCUS_SUBDIVISION	3.6%	3.6%
BACTEROIDES_AND_CYTOPHAGA	7.1%	7.1%
CHLOROFLEXUS_SUBDIVISION	3.6%	3.6%
CY.AURANTIACA_GROUP	7.1%	7.1%
CYANOBACTERIA	7.1%	7.1%
DELTA_SUBDIVISION	14.3%	14.3%
ENVIRONMENTAL_CLONE_WCHB1-41_SUBGROUP	7.1%	7.1%
FLX.LITORALIS_GROUP	3.6%	3.6%
GAMMA_SUBDIVISION	10.7%	10.7%
HIGH_G+C_BACTERIA	7.1%	7.1%
LEPTOSPIRILLUM_GROUP	3.6%	3.6%
MYCOPLASMA_AND_RELATIVES	3.6%	3.6%
PIRELLULA_GROUP	3.6%	3.6%
SPHINGOBACTERIUM_GROUP	3.6%	3.6%
SPIROCHAETA-TREPONEMA-BORRELIA_SUBDIVISION	3.6%	3.6%
THERMOANAEROBACTER_AND_RELATIVES	3.6%	3.6%

EXAMPLE 3: Multitag Pyrosequence Analysis of Dysbiosis in IBD

Inflammatory Bowel Diseases (IBD or IBDs), namely ulcerative colitis (UC) and Crohn's disease (CD), are chronic, lifelong, relapsing illnesses, affecting close to 1 million Americans and costing approximately $2 billion per year to the US healthcare system. IBDs are of unknown cause, have no cure, and are increasing in incidence. The natural course of these diseases is characterized by periods of quiescence (inactive disease) interspersed with flare-ups (active disease). It is now widely accepted that flare-ups of IBD are due to a dysregulated inflammatory reaction to abnormal intestinal microflora dysbiosis), however.

Specific changes in the microflora of IBD patients that might cause these diseases remain unknown. Narrow searches for a single pathogen that causes IBD have been

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unsuccessful. (See Guarner and Malagelada 2003). Studies of small bacterial groups have yielded ambiguous results. (See Schultz and Sartor 2000). Only recently have studies of large sets of bacterial flora been attempted. (See Eckburg, Bik, et al. 2005),. Improving our knowledge about GI tract microflora has the potential to revolutionize IBD treatment. Development of real-time methods to study microfloral changes may lead to diagnostic tools to predict flare-ups, and to targeted, safe treatments for IBD.

The key requirement to understanding dysbiosis in polymicrobial diseases is for a method to interrogate widely the microflora in numerous control and disease samples to identify dynamic trends in species composition associated with health and disease progression. In classic community studies (Eckburg, Bik, et al. 2005) environmental samples are assayed independently and then the clone sequence data from specific classes/groups are statistically analyzed usually using some sort of averaging metric. This can be extremely costly, especially if the clone libraries are exhaustively analyzed (i.e., 10,000 clones per sample).

To improve throughput and reduce cost, Amplicon Length Heterogeneity PCR (ALH-PCR) has been used to study the gut microflora. It offers a rapid way of screening complex microbial communities, allowing for easy fingerprinting of microfloral changes. The LH-PCR fingerprinting is inexpensive and fast, with the ability to screen several hundred samples a day. It can be used as a routine survey tool to monitor the dynamics of natural soil microbial communities or to quickly identify samples of interest using PCO analysis. PCO analysis has been used to group samples with similar profiles, allowing them to be pooled for cloning and sequencing. This greatly reduces the cost of analyzing multiple samples, particularly when the analysis requires sequencing at least 300 random clones to identify bacterial components of the sample down to 1% representation in the total population (which is the resolution limit for ALH fingerprinting). Pooling similar samples before cloning and sequencing has proved to be quite robust. However, equal amounts of the PCR product from each sample must be pooled or the results will be skewed.

Multitag Pyrosequencing is a novel pyrosequencing technology that allows many community samples to be sequenced together at high coverage without the necessity for fingerprinting, cloning, or the purification and separation techniques required by conventional methods for analyzing microbial communities, as described herein above. Multitag sequencing is more efficient, faster, and less costly than other methods.

By way of illustration, Multitag Pyrosequencing can be carried out using a set of specific tags on the end of standard universal small ribosomal sub-unit ("SSU") rRNA

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primers (See Table 1). A different set of the tagged primers is used to amplify the SSU rRNA in each different environmental sample (Figure 2-Step 1). The PCR amplicons from all the samples are pooled. Emulsion PCR is performed and the amplicons arising from each molecule are captured on their respective beads. Following amplification, the beads are distributed into the wells of a picoliter plate (Figure 2-Step 2). The sequences, including the tagged sequences, of the amplicons on each bead are determined by pyrosequencing (Figure 2-Step 3). A PERL script or other suitable program is used to sort the sequence information using the tags and primer sequence as a key. Sequences with the same tags are identified thereby with their respective sample. The bacteria species in each sample then are identified by matching the SSU rRNA sequences to entries in the database of the Ribosomal Database Project (either RDP 8.1 or RDP 9.0). The normalized frequency with which a bacteria is thus identified in a given sample is indicative of its relative representation in the microbial community. Histograms based on these frequency determinations can be used for the non-parametric analysis of dysbiotic shifts involved in disease states.

For example, Figure 3 depicts the results of such an experiment in which six Control, ten Crohns, and eight Ulcerative colitis mucosal samples were analyzed by Multitag Pyrosequencing. E ach of the segments in the stacked histogram bars represents the normalized abundance of that specific taxa in a specific sample. In this experiment, identification of the taxa was performed using BLAST analysis of the RDP 8.1 database. It can be seen that some taxa (i.e. Bacillus fragilis subgroup and Rumanococcus gnavus subgroup) are present in the same abundance in both control and disease states. Other taxa, such as Clostridium leptum are more dominant in Ulcerative colitis, while others (i.e. the Glocothece glococapsa subgroup) are indicators of dysbiosis in the disease state.

However, the standard 454 Life Science process using a ligation step to link the emulsion PCR adapters to the PCR amplicons and produces numerous artifacts in the quantitation of the abundances of each taxa in the samples. In the results displayed in Figure 3, we algorithmically removed chimeras, reverse reads and truncated products and filtered the data to remove all taxa that were represented by less than 5% abundance. Only then were we able to see a correlation with disease state and specific microbial taxa.

EXAMPLE 4: Distortion of the distribution of components of a microbial community by directly ligating Emulsion PCR adapters onto PCR amplicons.

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In one experiment we used tagged PCR primers to amplify the components in duplicate microbial community samples, ligated the Emulsion PCR adapters to these samples, and then subjected these samples to separate pyrosequencing runs. The amplicons are routinely run on an Agilent Bioanalyzer system before and after ligation to quantitate the mixture before emulsion PCR. Figure 4 depicts a sample run on the Bioanalyzer before and after direct ligation and clearly shows that the ligation step has drastically altered the distribution of the amplicons.

Additionally, we compared the normalized abundances of the component taxa identified by the multitag process after direct ligation of the Emulsion PCR adapters. In this experiment, identification of the taxa was performed using a Bayesian analysis of the RDP 9.0 database. We can se in Figure 5 that abundances of the forward and reverse primers for various taxa are different within a sample and between duplicate samples. In several cases, we are missing entire families in the comparison between duplicates. Table 3 summarizes the differences between the forward primers and the reverse primers of the duplicate samples and it is clearly stochastic with no predictable pattern. We hypothesize that this differential ligation efficiency could be due to a number of factors such as internal structure in the amplicons or biases in the terminal nucleotide of either the adapter or amplicon.

Table 3 Duplicate Sample Analysis

RDP 9.0 FAMILY	FORWARD PRIMERS RATIOS	REVERSE PRIMER RATIOS
Acidaminococcaceae	544.6%	195.0%
Actinomycetales	144.0%	116.5%
Bacteroidaceae	119.9%	124.5%
Clostridiaceae	97.5%	99.4%
Comamonadaceae	198.0%	
Coriobacteriales	181.5%	141.5%
Enterobacteriaceae	4.2%	
Eubacteriaceae	88.0%	87.5%
Flavobacteriaceae	34.9%	
Incertae sedis 9	106.4%	143.0%
Lachnospiraceae	176.8%	113.1%
Peptococcaceae		91.0%
Peptostreptococcaceae	94.7%	115.4%
Porphyromonadaceae	99.0%	97.3%
Prevotellaceae	264.0%	88.1%
Rikenellaceae	212.2%	106.1%
Streptococcaceae	74.3%	60.7%

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What is claimed is:

1. A multiplex method for the quantitative determination of polynucleotides in two or more samples, comprising:

hybridizing a first primer to polynucleotides in a first sample, said first primer comprising a first tag sequence and a first probe sequence specific for a first target sequence, wherein said first target sequence is 3' to a variable genetic region;

elongating primer templates formed thereby to form a first population of tagged polynucleotides comprising: said first primer including said first tag sequence; and sequences of said variable genetic region;

hybridizing a second primer to polynucleotides in a second sample, said second primer comprising a second tag sequence and a second probe sequence specific for a second target sequence, wherein said second target sequence is 3' to the same variable genetic region as said first target sequence, wherein further said second probe sequence may be the same as or different from said first probe sequence;

elongating primer templates formed thereby to form a second population of tagged polynucleotides comprising: said second primer including said second tag sequence; and sequences of said variable genetic region;

mixing said first and second populations together;

determining sequences of polynucleotides comprising tag sequences and the sequences of the variable genetic element in said mixture;

from the tag sequences comprised in the polynucleotide sequences thus determined identifying the sample in which polynucleotide sequences occurred;

from the sequences of the variable genetic region comprised in the polynucleotide sequences thus determined identifying particular variants of said variable genetic element;

from this information determining the number of time one or more given variants occur in each sample, and

from the number for each variant in the polynucleotides thus determined, quantifying said polynucleotides in said samples;

wherein said sequences are determined without Southern blot transfer and/or without size-separating primer extension products and/or without electrophoresis.

2. A multiplex method for the quantitative determination of polynucleotides in two or more samples, comprising:

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hybridizing a first primer pair to polynucleotides in a first sample, the first primer of said first primer pair comprising a first tag sequence and a first probe sequences specific for a first target sequence and the second primer of said first primer pair comprising a second tag sequence and a second probe sequence specific for a second target sequence, wherein the first and the second probe sequences flank and hybridize to opposite strands of a variable genetic region;

elongating primer templates formed thereby to from a first population of tagged polynucleotides, each of said polynucleotides comprising: (a) the sequence of said first primer of said first primer pair, a sequence of said variable genetic region, and a sequence complementary to the sequence of said second primer of said first primer pair or (b) a sequence complementary to the sequence of said first primer of said first primer pair, a sequence of said variable genetic region and the sequence of said second primer of said first primer pair;

hybridizing a second primer pair to polynucleotides in a second sample, the first primer of said second primer pair comprising a third tag sequence and said first probe sequences specific for said first target sequence and the second primer of said second primer pair comprising a fourth tag sequence and said second probe sequence specific for said second target sequence;

elongating primer templates formed thereby to from a second population of tagged polynucleotides, each of said polynucleotides comprising: (a) the sequence of said first primer of said second primer pair, a sequence of said variable genetic region, and a sequence complementary to the sequence of said second primer of said second primer pair or (b) a sequence complementary to the sequence of said first primer of said second primer pair, a sequence of said variable genetic region and the sequence of said second primer of said second primer pair;

mixing said first and second populations together;

determining sequences of polynucleotides in said mixture, comprising the tag sequences and the variable genetic element;

from the tag sequences comprised in the polynucleotide sequences thus determined identifying the sample in which polynucleotide sequences occurred;

from the sequences of the variable genetic region comprised in the polynucleotide sequences thus determined identifying particular variants of said variable genetic element;

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from this information determining the number of times given variants occur in each sample, and

from the number for each variant in the polynucleotides thus determined, quantifying said polynucleotides in said samples.

wherein said sequences are determined without Southern blot transfer and/or without size-separating primer extension products and/or without electrophoresis.

3. A method according to claim 2, wherein the number of said polynucleotides in said first sample is any of 5, 10, 25, 50, 100, 150, 200, 250, 500, 1,000, 1,500, 2,000, 2,500, 5,000, 7,500, 10,000, 12,500, 15,000, 17,500, 20,000, 25,000, 30,000, 35,000, 40,000, 50,000, 75,000, 100,000, 150,000, 200,000, 250,000, 500,000, 1,000,000 or more, and the number of said polynucleotides in said second sample is any of 5, 10, 25, 50, 100, 150, 200, 250, 500, 1,000, 1,500, 2,000, 2,500, 5,000, 7,500, 10,000, 12,500, 15,000, 17,500, 20,000, 25,000, 30,000, 35,000, 40,000, 50,000, 75,000, 100,000, 150,000, 200,000, 250,000, 500,000, 1,000,000 or more.

4. A method according to claim 2, wherein the number of said samples and of said different tags therefor is 5, 10, 15, 20, 25, 50, 75, 100, 150, 200, 250, 500, 1,000, 2,500, 5,000, 10,000 or more.

5. A method according to claim 2, wherein the tags are nucleotide sequences that are 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36 nucleotides long or any combination thereof.

6. A method according to claim 2, wherein said tags are comprised in primers for amplification and are incorporated into said polynucleotides by amplification using said primers.

7. A method according to claim 6, wherein the primer for amplification comprises a sequence for PCR amplification, linear amplification, transcriptional amplification, rolling circle replication, or QB replication.

8. A method according to claim 7, wherein the primer for amplification comprises a sequence for PCR amplification.

9. A method according to claim 2, wherein each of said polynucleotides is disposed individually on a bead isolated from other polynucleotides.

10. A method according to claim 2, wherein each of said polynucleotides is disposed individually on a bead isolated from other said polynucleotides, is amplified while disposed therein, and the amplification products thereof also are disposed on said bead.

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11. A method according to claim 2, wherein each of said polynucleotides is disposed individually on a bead isolated from other said polynucleotides, is amplified while disposed therein, the amplification products thereof also are disposed on said bead, and each said bead is disposed individually in a well isolated from other said beads.

12. A method according to claim 2, wherein the sequences are determined by pyrosequencing.

13. A method according to claim 2, wherein said samples are biological samples, each comprising one or more species.

14. A method according to claim 2, wherein variants of the variable genetic region are specific to particular organisms.

15. A method according to claim 2, wherein said variable genetic region comprises a variable 16S rRNA sequence, a variable 18S rRNA sequence, a variable rRNA ITS sequence, a mitochondrial sequence, a microsatellite sequence, a metabolic enzyme sequence, and/or a genetic disease sequence.

16. A method according to claim 2, wherein the samples are microbial community samples.

17. A method according to claim 2, wherein the samples are microbial community samples for clinical analysis of a patient.

18. A method according to claim 2, wherein the samples are microbial community environmental samples.

19. A method according to claim 2, wherein the samples are microbial community soil samples.

20. A method according to claim 2, wherein the samples are microbial community water samples.

21. A method according to claim 2, wherein the samples are samples for SNP analysis.

22. A method according to claim 2, wherein the samples are samples for genotyping.

23. A kit comprising a plurality of two or more primers, each primer in said plurality comprising a tag sequence and a probe sequence specific to a target sequence, wherein:

(A) in each of said primers the probe sequence is 3' to the tag sequence, but not necessarily adjacent thereto;

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(B) in each of said primers: the tag sequence is different from the tag sequence of the other in the plurality; the tag sequence is not the complementary sequence to any other tag sequence in the plurality; the tag sequence does not contain any homodinucleotide sequences; the junction sequences between the tag sequence and the adjacent parts of the primer, if any, is not a homodinucleotide sequence;

(C) in each of said primers the probe sequence is complementary to the target sequence and the target sequence is located 3' to a variable genetic region, and

(D) each of said primers is disposed separately from the others in containers in said kit.

24. A kit according to claim 23, wherein each of said primers further comprises a priming sequence 5' to the tag sequence but not necessarily adjacent thereto, and the priming sequence is the same in all of said primers, said kit further comprising a primer complimentary to and effective for polymerization from said priming sequence.

25. A kit comprising a plurality of two or more primers pairs, each primer in said plurality comprising a tag sequence and a probe sequence specific to a target sequence, wherein:

(A) in each of said primer the probe sequence is 3' to the tag sequence, but not necessarily adjacent thereto;

(B) in each of said primers: the tag sequence is different from the tag sequence of the other in the plurality; the tag sequence is not the complementary sequence to any other tag sequence in the plurality; the tag sequence does not contain any homodinucleotide sequences; the junction sequences between the tag sequence and the adjacent parts of the primer, if any, is not a homodinucleotide sequence;

(C) in each of said primers the probe sequence is complementary to the target sequence,

(D) in each primer pair the probe sequences are specific to target sequences that flank a variable genetic region;

(E) each of said primers is disposed separately from the others in said kit.

26. A kit according to claim 25, wherein the primers further comprise a priming sequence 5' to the tag sequence but not necessarily adjacent thereto, the priming sequence either is the same in all the primers, or one member of each pair has the same first priming sequence and the second member of each pair has the same second priming sequence, said kit further comprising disposed separately from one another in one or more containers one or

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more primers complementary to and effective for elongation from said priming sequence or sequences.

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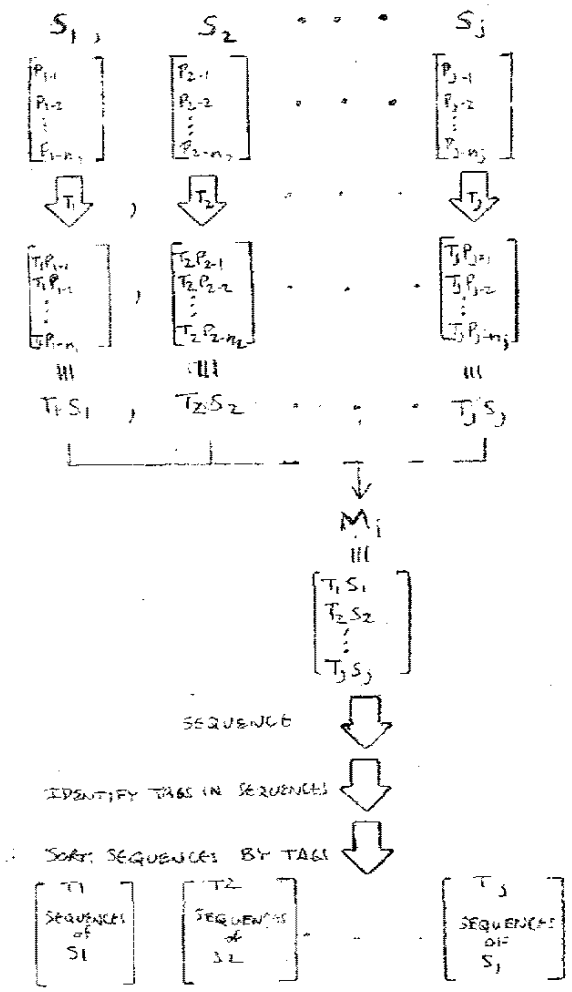

SEQUENCE
IDENTIFY TAGS IN SEQUENCES
SORT SEQUENCES BY TAGS

Figure 1

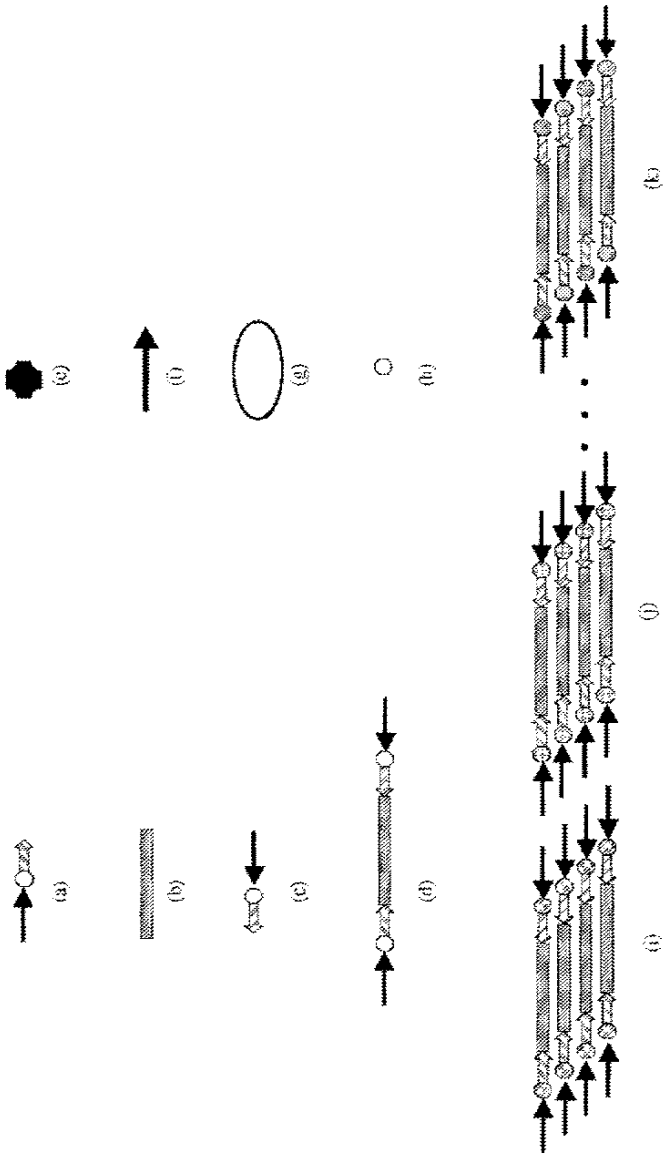


Figure 2A

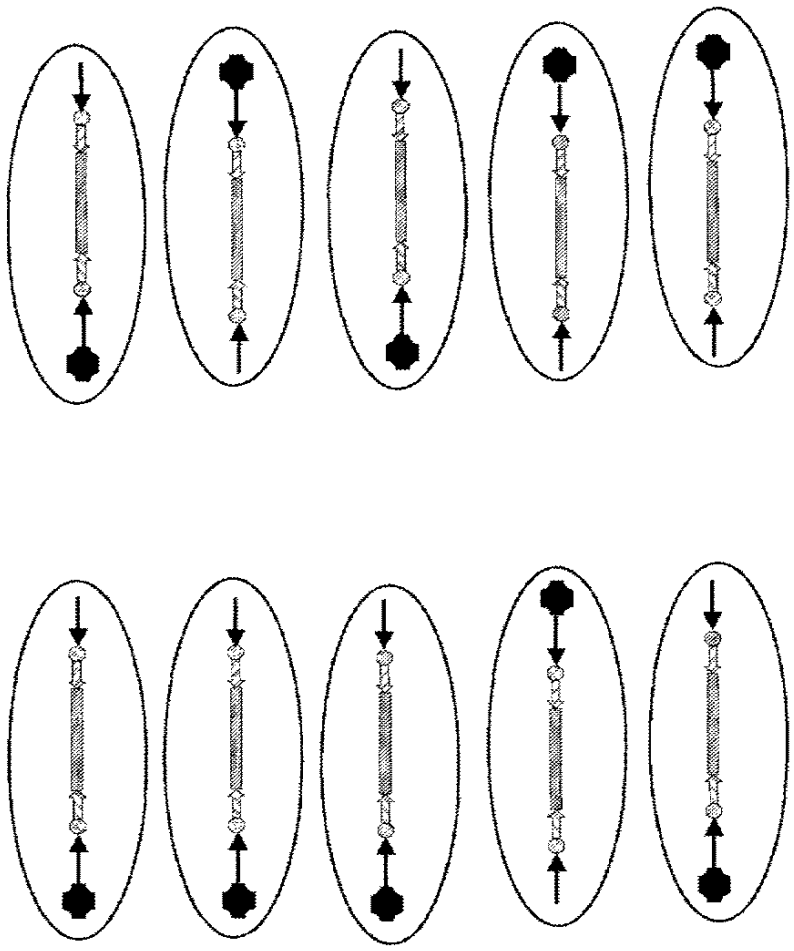


Figure 2 B

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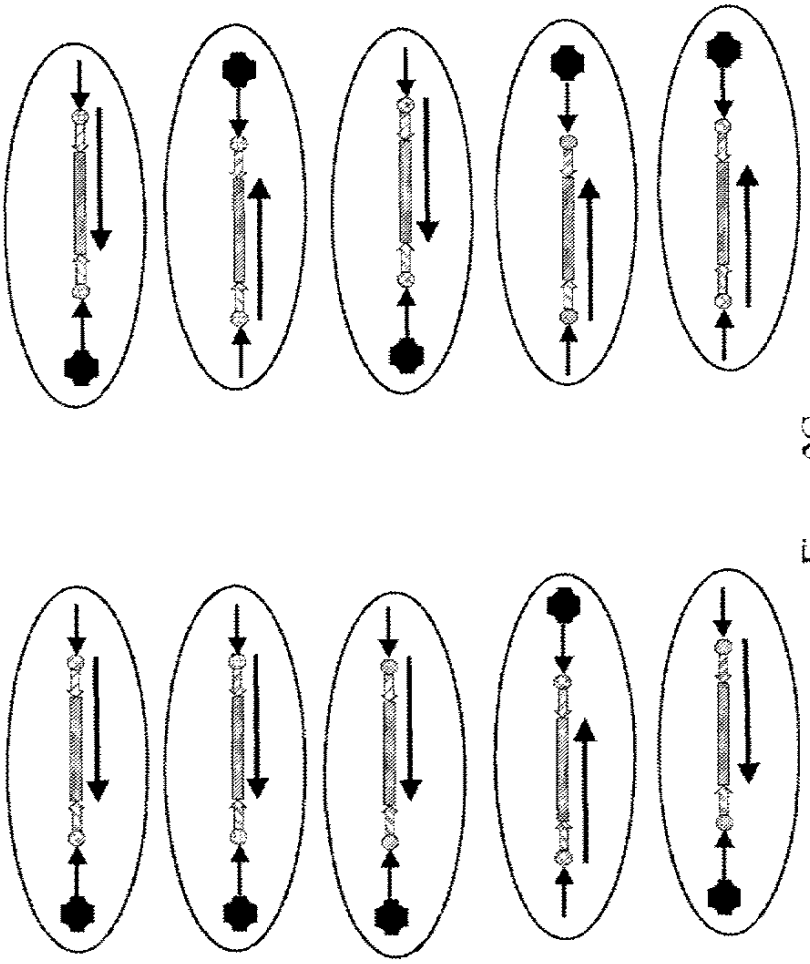


Figure 2C

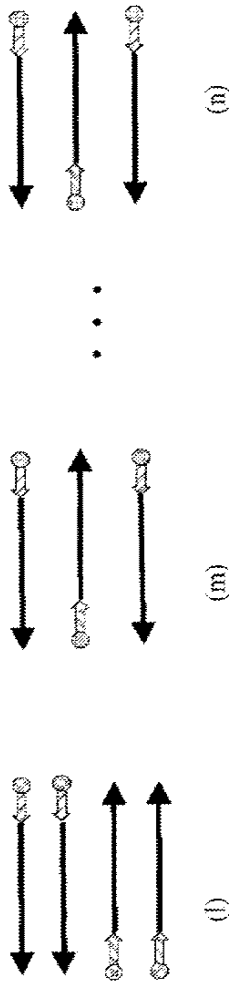


Figure 2D

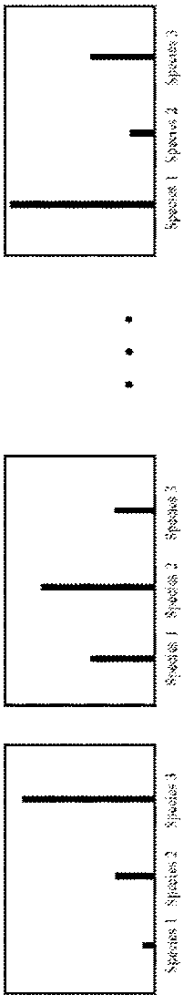


Figure 2E

FIGURE 3

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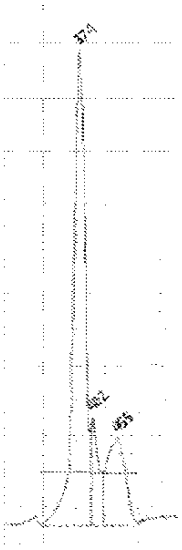


Figure 4A

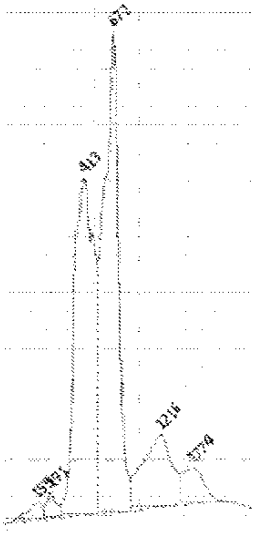


Figure 4B

FAMILY	Forward Primer	Reverse Primer	RATIO	Forward Primer	Reverse Primer	RATIO
	ALD309FO	ALD309RO	Forward/Reverse	ALD309FP	ALD309RP	Forward/Reverse
Acidaminococcaceae	0.8%	0.9%	**88.5%**	0.2%	0.5%	**31.7%**
Actinomycetales	2.4%	2.0%	**120.7%**	1.7%	1.7%	**97.6%**
Bacteroidaceae	58.4%	62.8%	**93.1%**	48.7%	50.5%	**96.6%**
Brucellaceae				7.5%	3.1%	**239.1%**
Burkholderiaceae				0.4%	0.2%	**184.9%**
Clostridiaceae	14.5%	9.6%	**150.7%**	14.9%	9.7%	**153.7%**
Comamonadaceae	0.3%			0.2%	0.3%	**55.5%**
Coriobacteriales	0.8%	0.9%	**94.8%**	0.5%	0.6%	**73.9%**
Enterobacteriaceae	0.2%			3.6%	2.9%	**124.1%**
Eubacteriaceae	1.8%	1.6%	**115.9%**	2.1%	1.8%	**115.2%**
Flavobacteriaceae	0.5%			1.3%	2.1%	**62.9%**
Incertae sedis 9	3.3%	4.2%	**78.6%**	3.1%	2.9%	**105.6%**
Lachnospiraceae	5.7%	8.7%	**65.6%**	3.2%	7.7%	**42.0%**
Lactobacillaceae				1.3%	0.5%	**269.4%**
Peptococcaceae		0.3%		0.2%	0.3%	**83.2%**
Peptostreptococcaceae	3.3%	3.3%	**102.1%**	3.5%	2.8%	**124.4%**
Phyllobacteriaceae				1.6%	6.2%	**26.2%**
Porphyromonadaceae	2.1%	2.0%	**109.0%**	2.2%	2.0%	**107.1%**
Prevotellaceae	3.0%	2.0%	**155.7%**	1.2%	2.2%	**52.0%**
Rikenellaceae	1.1%	0.9%	**129.3%**	0.5%	0.8%	**64.7%**
Streptococcaceae	0.5%	0.3%	**181.1%**	0.6%	0.4%	**147.9%**

FIGURE 5

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1	25	49	73	97	121	145	169	193
AGACAG	AGTGAG	ATGCAG	CATACA	CGCTAG	GCGCAG	GTCACA	TACTAG	TCAGAG
AGACGT	AGTGCA	ATGCGT	CATAGT	CGCTCA	GCGCGT	GTCAGT	TACTCA	TCAGCA
AGACTC	AGTGTC	ATGCTC	CATATC	CGCTGT	GCGCTC	GTCATC	TACTGT	TCAGTC
AGAGAG	ATACAG	ATGTAG	CATCAG	CGTACA	GCGTAG	GTCGAG	TAGACA	TCATAG
AGAGCA	ATACGT	ATGTCA	CATCGT	CGTAGT	GCGTCA	GTCGCA	TAGAGT	TCATCA
AGAGTC	ATACTC	ATGTGT	CATCTC	CGTATC	GCGTGT	GTCGTC	TAGATC	TCATGT
AGATAG	ATAGAG	CACACA	CATGAG	CGTCAG	GCTACA	GTCTAG	TAGCAG	TCGACA
AGATCA	ATAGCA	CACAGT	CATGCA	CGTCGT	GCTAGT	GTCTCA	TAGCGT	TCGAGT
AGATGT	ATAGTC	CACATC	CATGTC	CGTCTC	GCTATC	GTCTGT	TAGCTC	TCGATC
AGCACA	ATATAG	CACGAG	CGACAG	CGTGAG	GCTCAG	GTGACA	TAGTAG	TCGCAG
AGCAGT	ATATCA	CACGCA	CGACGT	CGTGCA	GCTCGT	GTGAGT	TAGTCA	TCGCGT
AGCATC	ATATGT	CACGTC	CGACTC	CGTGTC	GCTCTC	GTGATC	TAGTGT	TCGCTC
AGCGAG	ATCACA	CACTAG	CGAGAG	GCACAG	GCTGAG	GTGCAG	TATACA	TCGTAG
AGCGCA	ATCAGT	CACTCA	CGAGCA	GCACGT	GCTGCA	GTGCGT	TATAGT	TCGTCA
AGCGTC	ATCATC	CACTGT	CGAGTC	GCACTC	GCTGTC	GTGCTC	TATATC	TCGTGT
AGCTAG	ATCGAG	CAGACA	CGATAG	GCAGAG	GTACAG	GTGTAG	TATCAG	TCTACA
AGCTCA	ATCGCA	CAGAGT	CGATCA	GCAGCA	GTACGT	GTGTCA	TATCGT	TCTAGT
AGCTGT	ATCGTC	CAGATC	CGATGT	GCAGTC	GTACTC	GTGTGT	TATCTC	TCTATC
AGTACA	ATCTAG	CAGCAG	CGCACA	GCATAG	GTAGAG	TACACA	TATGAG	TCTCAG
AGTAGT	ATCTCA	CAGCGT	CGCAGT	GCATCA	GTAGCA	TACAGT	TATGCA	TCTCGT
AGTATC	ATCTGT	CAGCTC	CGCATC	GCATGT	GTAGTC	TACATC	TATGTC	TCTCTC
AGTCAG	ATGACA	CAGTAG	CGCGAG	GCGACA	GTATAG	TACGAG	TCACAG	TCTGAG
AGTCGT	ATGAGT	CAGTCA	CGCGCA	GCGAGT	GTATCA	TACGCA	TCACGT	TCTGCA
AGTCTC	ATGATC	CAGTGT	CGCGTC	GCGATC	GTATGT	TACGTC	TCACTC	TCTGTC
24	48	72	96	120	144	168	192	216

FIGURE 6

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FIGURE 7A

FORWARD TAGGED ADAPTER PRIMER

Primer Pair	ADAPTER A	TAG	Forward 16S rRNA Primer
1	GCCTCCCTCGCGCCATCAG	AGACGT	AGAGTTTGATCMTGGCTCAG
2	GCCTCCCTCGCGCCATCAG	AGACTC	AGAGTTTGATCMTGGCTCAG
3	GCCTCCCTCGCGCCATCAG	AGAGTC	AGAGTTTGATCMTGGCTCAG
4	GCCTCCCTCGCGCCATCAG	AGATGT	AGAGTTTGATCMTGGCTCAG
5	GCCTCCCTCGCGCCATCAG	AGCAGT	AGAGTTTGATCMTGGCTCAG
6	GCCTCCCTCGCGCCATCAG	AGCATC	AGAGTTTGATCMTGGCTCAG
7	GCCTCCCTCGCGCCATCAG	AGCGTC	AGAGTTTGATCMTGGCTCAG
8	GCCTCCCTCGCGCCATCAG	AGCTGT	AGAGTTTGATCMTGGCTCAG
9	GCCTCCCTCGCGCCATCAG	AGTAGT	AGAGTTTGATCMTGGCTCAG
10	GCCTCCCTCGCGCCATCAG	AGTATC	AGAGTTTGATCMTGGCTCAG
11	GCCTCCCTCGCGCCATCAG	AGTCGT	AGAGTTTGATCMTGGCTCAG
12	GCCTCCCTCGCGCCATCAG	AGTCTC	AGAGTTTGATCMTGGCTCAG
13	GCCTCCCTCGCGCCATCAG	AGTGTC	AGAGTTTGATCMTGGCTCAG
14	GCCTCCCTCGCGCCATCAG	ATACGT	AGAGTTTGATCMTGGCTCAG
15	GCCTCCCTCGCGCCATCAG	ATACTC	AGAGTTTGATCMTGGCTCAG
16	GCCTCCCTCGCGCCATCAG	ATAGTC	AGAGTTTGATCMTGGCTCAG
17	GCCTCCCTCGCGCCATCAG	ATATGT	AGAGTTTGATCMTGGCTCAG
18	GCCTCCCTCGCGCCATCAG	ATCAGT	AGAGTTTGATCMTGGCTCAG
19	GCCTCCCTCGCGCCATCAG	ATCATC	AGAGTTTGATCMTGGCTCAG
20	GCCTCCCTCGCGCCATCAG	ATCGTC	AGAGTTTGATCMTGGCTCAG
21	GCCTCCCTCGCGCCATCAG	ATCTGT	AGAGTTTGATCMTGGCTCAG
22	GCCTCCCTCGCGCCATCAG	ATGAGT	AGAGTTTGATCMTGGCTCAG
23	GCCTCCCTCGCGCCATCAG	ATGATC	AGAGTTTGATCMTGGCTCAG
24	GCCTCCCTCGCGCCATCAG	ATGCGT	AGAGTTTGATCMTGGCTCAG
25	GCCTCCCTCGCGCCATCAG	ATGCTC	AGAGTTTGATCMTGGCTCAG
26	GCCTCCCTCGCGCCATCAG	ATGTGT	AGAGTTTGATCMTGGCTCAG
27	GCCTCCCTCGCGCCATCAG	CACAGT	AGAGTTTGATCMTGGCTCAG
28	GCCTCCCTCGCGCCATCAG	CACATC	AGAGTTTGATCMTGGCTCAG
29	GCCTCCCTCGCGCCATCAG	CACGTC	AGAGTTTGATCMTGGCTCAG
30	GCCTCCCTCGCGCCATCAG	CACTGT	AGAGTTTGATCMTGGCTCAG
31	GCCTCCCTCGCGCCATCAG	CAGAGT	AGAGTTTGATCMTGGCTCAG
32	GCCTCCCTCGCGCCATCAG	CAGATC	AGAGTTTGATCMTGGCTCAG
33	GCCTCCCTCGCGCCATCAG	CAGCGT	AGAGTTTGATCMTGGCTCAG
34	GCCTCCCTCGCGCCATCAG	CAGCTC	AGAGTTTGATCMTGGCTCAG
35	GCCTCCCTCGCGCCATCAG	CAGTGT	AGAGTTTGATCMTGGCTCAG
36	GCCTCCCTCGCGCCATCAG	CATAGT	AGAGTTTGATCMTGGCTCAG
37	GCCTCCCTCGCGCCATCAG	CATATC	AGAGTTTGATCMTGGCTCAG
38	GCCTCCCTCGCGCCATCAG	CATCGT	AGAGTTTGATCMTGGCTCAG
39	GCCTCCCTCGCGCCATCAG	CATCTC	AGAGTTTGATCMTGGCTCAG
40	GCCTCCCTCGCGCCATCAG	CATGTC	AGAGTTTGATCMTGGCTCAG
41	GCCTCCCTCGCGCCATCAG	CGACGT	AGAGTTTGATCMTGGCTCAG
42	GCCTCCCTCGCGCCATCAG	CGACTC	AGAGTTTGATCMTGGCTCAG
43	GCCTCCCTCGCGCCATCAG	CGAGTC	AGAGTTTGATCMTGGCTCAG
44	GCCTCCCTCGCGCCATCAG	CGATGT	AGAGTTTGATCMTGGCTCAG
45	GCCTCCCTCGCGCCATCAG	CGCAGT	AGAGTTTGATCMTGGCTCAG
46	GCCTCCCTCGCGCCATCAG	CGCATC	AGAGTTTGATCMTGGCTCAG
47	GCCTCCCTCGCGCCATCAG	CGCGTC	AGAGTTTGATCMTGGCTCAG
48	GCCTCCCTCGCGCCATCAG	CGCTGT	AGAGTTTGATCMTGGCTCAG
49	GCCTCCCTCGCGCCATCAG	CGTAGT	AGAGTTTGATCMTGGCTCAG
50	GCCTCCCTCGCGCCATCAG	CGTATC	AGAGTTTGATCMTGGCTCAG

FIGURE 7B

FORWARD TAGGED ADAPTER PRIMER

Primer Pair	ADAPTER A	TAG	Forward 16S rRNA Primer
51	GCCTCCCTCGCGCCATCAG	CGTCGT	AGAGTTTGATCMTGGCTCAG
52	GCCTCCCTCGCGCCATCAG	CGTCTC	AGAGTTTGATCMTGGCTCAG
53	GCCTCCCTCGCGCCATCAG	CGTGCA	AGAGTTTGATCMTGGCTCAG
54	GCCTCCCTCGCGCCATCAG	CGTGTC	AGAGTTTGATCMTGGCTCAG
55	GCCTCCCTCGCGCCATCAG	TACAGT	AGAGTTTGATCMTGGCTCAG
56	GCCTCCCTCGCGCCATCAG	TACATC	AGAGTTTGATCMTGGCTCAG
57	GCCTCCCTCGCGCCATCAG	TACGTC	AGAGTTTGATCMTGGCTCAG
58	GCCTCCCTCGCGCCATCAG	TACTGT	AGAGTTTGATCMTGGCTCAG
59	GCCTCCCTCGCGCCATCAG	TAGAGT	AGAGTTTGATCMTGGCTCAG
60	GCCTCCCTCGCGCCATCAG	TAGATC	AGAGTTTGATCMTGGCTCAG
61	GCCTCCCTCGCGCCATCAG	TAGCGT	AGAGTTTGATCMTGGCTCAG
62	GCCTCCCTCGCGCCATCAG	TAGCTC	AGAGTTTGATCMTGGCTCAG
63	GCCTCCCTCGCGCCATCAG	TAGTGT	AGAGTTTGATCMTGGCTCAG
64	GCCTCCCTCGCGCCATCAG	TATAGT	AGAGTTTGATCMTGGCTCAG
65	GCCTCCCTCGCGCCATCAG	TATATC	AGAGTTTGATCMTGGCTCAG
66	GCCTCCCTCGCGCCATCAG	TATCGT	AGAGTTTGATCMTGGCTCAG
67	GCCTCCCTCGCGCCATCAG	TATCTC	AGAGTTTGATCMTGGCTCAG
68	GCCTCCCTCGCGCCATCAG	TATGTC	AGAGTTTGATCMTGGCTCAG
69	GCCTCCCTCGCGCCATCAG	TCACGT	AGAGTTTGATCMTGGCTCAG
70	GCCTCCCTCGCGCCATCAG	TCACTC	AGAGTTTGATCMTGGCTCAG
71	GCCTCCCTCGCGCCATCAG	TCAGTC	AGAGTTTGATCMTGGCTCAG
72	GCCTCCCTCGCGCCATCAG	TCATGT	AGAGTTTGATCMTGGCTCAG
73	GCCTCCCTCGCGCCATCAG	TCGAGT	AGAGTTTGATCMTGGCTCAG
74	GCCTCCCTCGCGCCATCAG	TCGATC	AGAGTTTGATCMTGGCTCAG
75	GCCTCCCTCGCGCCATCAG	TCGCGT	AGAGTTTGATCMTGGCTCAG
76	GCCTCCCTCGCGCCATCAG	TCGCTC	AGAGTTTGATCMTGGCTCAG
77	GCCTCCCTCGCGCCATCAG	TCGTGT	AGAGTTTGATCMTGGCTCAG
78	GCCTCCCTCGCGCCATCAG	TCTAGT	AGAGTTTGATCMTGGCTCAG
79	GCCTCCCTCGCGCCATCAG	TCTATC	AGAGTTTGATCMTGGCTCAG
80	GCCTCCCTCGCGCCATCAG	TCTCGT	AGAGTTTGATCMTGGCTCAG
81	GCCTCCCTCGCGCCATCAG	TCTCTC	AGAGTTTGATCMTGGCTCAG
82	GCCTCCCTCGCGCCATCAG	TCTGTC	AGAGTTTGATCMTGGCTCAG
83	GCCTCCCTCGCGCCATCAG	AGACAG	AGAGTTTGATCMTGGCTCAG
84	GCCTCCCTCGCGCCATCAG	AGAGAG	AGAGTTTGATCMTGGCTCAG
85	GCCTCCCTCGCGCCATCAG	AGATAG	AGAGTTTGATCMTGGCTCAG
86	GCCTCCCTCGCGCCATCAG	AGCGAG	AGAGTTTGATCMTGGCTCAG
87	GCCTCCCTCGCGCCATCAG	AGCTAG	AGAGTTTGATCMTGGCTCAG
88	GCCTCCCTCGCGCCATCAG	AGTCAG	AGAGTTTGATCMTGGCTCAG
89	GCCTCCCTCGCGCCATCAG	AGTGAG	AGAGTTTGATCMTGGCTCAG
90	GCCTCCCTCGCGCCATCAG	ATACAG	AGAGTTTGATCMTGGCTCAG
91	GCCTCCCTCGCGCCATCAG	ATAGAG	AGAGTTTGATCMTGGCTCAG
92	GCCTCCCTCGCGCCATCAG	ATATAG	AGAGTTTGATCMTGGCTCAG
93	GCCTCCCTCGCGCCATCAG	ATCGAG	AGAGTTTGATCMTGGCTCAG
94	GCCTCCCTCGCGCCATCAG	ATCTAG	AGAGTTTGATCMTGGCTCAG
95	GCCTCCCTCGCGCCATCAG	ATGCAG	AGAGTTTGATCMTGGCTCAG
96	GCCTCCCTCGCGCCATCAG	ATGTAG	AGAGTTTGATCMTGGCTCAG

FIGURE 7C

REVERSE TAGGED ADAPTER PRIMER

Primer Pair	ADAPTER B	TAG	Reverse 16S rRNAPrimer
1	GCCTTGCCAGCCCGCTCAG	AGACGT	GCTGCCTCCCGTAGGAGT
2	GCCTTGCCAGCCCGCTCAG	AGACTC	GCTGCCTCCCGTAGGAGT
3	GCCTTGCCAGCCCGCTCAG	AGAGTC	GCTGCCTCCCGTAGGAGT
4	GCCTTGCCAGCCCGCTCAG	AGATGT	GCTGCCTCCCGTAGGAGT
5	GCCTTGCCAGCCCGCTCAG	AGCAGT	GCTGCCTCCCGTAGGAGT
6	GCCTTGCCAGCCCGCTCAG	AGCATC	GCTGCCTCCCGTAGGAGT
7	GCCTTGCCAGCCCGCTCAG	AGCGTC	GCTGCCTCCCGTAGGAGT
8	GCCTTGCCAGCCCGCTCAG	AGCTGT	GCTGCCTCCCGTAGGAGT
9	GCCTTGCCAGCCCGCTCAG	AGTAGT	GCTGCCTCCCGTAGGAGT
10	GCCTTGCCAGCCCGCTCAG	AGTATC	GCTGCCTCCCGTAGGAGT
11	GCCTTGCCAGCCCGCTCAG	AGTCGT	GCTGCCTCCCGTAGGAGT
12	GCCTTGCCAGCCCGCTCAG	AGTCTC	GCTGCCTCCCGTAGGAGT
13	GCCTTGCCAGCCCGCTCAG	AGTGTC	GCTGCCTCCCGTAGGAGT
14	GCCTTGCCAGCCCGCTCAG	ATACGT	GCTGCCTCCCGTAGGAGT
15	GCCTTGCCAGCCCGCTCAG	ATACTC	GCTGCCTCCCGTAGGAGT
16	GCCTTGCCAGCCCGCTCAG	ATAGTC	GCTGCCTCCCGTAGGAGT
17	GCCTTGCCAGCCCGCTCAG	ATATGT	GCTGCCTCCCGTAGGAGT
18	GCCTTGCCAGCCCGCTCAG	ATCAGT	GCTGCCTCCCGTAGGAGT
19	GCCTTGCCAGCCCGCTCAG	ATCATC	GCTGCCTCCCGTAGGAGT
20	GCCTTGCCAGCCCGCTCAG	ATCGTC	GCTGCCTCCCGTAGGAGT
21	GCCTTGCCAGCCCGCTCAG	ATCTGT	GCTGCCTCCCGTAGGAGT
22	GCCTTGCCAGCCCGCTCAG	ATGAGT	GCTGCCTCCCGTAGGAGT
23	GCCTTGCCAGCCCGCTCAG	ATGATC	GCTGCCTCCCGTAGGAGT
24	GCCTTGCCAGCCCGCTCAG	ATGCGT	GCTGCCTCCCGTAGGAGT
25	GCCTTGCCAGCCCGCTCAG	ATGCTC	GCTGCCTCCCGTAGGAGT
26	GCCTTGCCAGCCCGCTCAG	ATGTGT	GCTGCCTCCCGTAGGAGT
27	GCCTTGCCAGCCCGCTCAG	CACAGT	GCTGCCTCCCGTAGGAGT
28	GCCTTGCCAGCCCGCTCAG	CACATC	GCTGCCTCCCGTAGGAGT
29	GCCTTGCCAGCCCGCTCAG	CACGTC	GCTGCCTCCCGTAGGAGT
30	GCCTTGCCAGCCCGCTCAG	CACTGT	GCTGCCTCCCGTAGGAGT
31	GCCTTGCCAGCCCGCTCAG	CAGAGT	GCTGCCTCCCGTAGGAGT
32	GCCTTGCCAGCCCGCTCAG	CAGATC	GCTGCCTCCCGTAGGAGT
33	GCCTTGCCAGCCCGCTCAG	CAGCGT	GCTGCCTCCCGTAGGAGT
34	GCCTTGCCAGCCCGCTCAG	CAGCTC	GCTGCCTCCCGTAGGAGT
35	GCCTTGCCAGCCCGCTCAG	CAGTGT	GCTGCCTCCCGTAGGAGT
36	GCCTTGCCAGCCCGCTCAG	CATAGT	GCTGCCTCCCGTAGGAGT
37	GCCTTGCCAGCCCGCTCAG	CATATC	GCTGCCTCCCGTAGGAGT
38	GCCTTGCCAGCCCGCTCAG	CATCGT	GCTGCCTCCCGTAGGAGT
39	GCCTTGCCAGCCCGCTCAG	CATCTC	GCTGCCTCCCGTAGGAGT
40	GCCTTGCCAGCCCGCTCAG	CATGTC	GCTGCCTCCCGTAGGAGT
41	GCCTTGCCAGCCCGCTCAG	CGACGT	GCTGCCTCCCGTAGGAGT
42	GCCTTGCCAGCCCGCTCAG	CGACTC	GCTGCCTCCCGTAGGAGT
43	GCCTTGCCAGCCCGCTCAG	CGAGTC	GCTGCCTCCCGTAGGAGT
44	GCCTTGCCAGCCCGCTCAG	CGATGT	GCTGCCTCCCGTAGGAGT
45	GCCTTGCCAGCCCGCTCAG	CGCAGT	GCTGCCTCCCGTAGGAGT
46	GCCTTGCCAGCCCGCTCAG	CGCATC	GCTGCCTCCCGTAGGAGT
47	GCCTTGCCAGCCCGCTCAG	CGCGTC	GCTGCCTCCCGTAGGAGT
48	GCCTTGCCAGCCCGCTCAG	CGCTGT	GCTGCCTCCCGTAGGAGT
49	GCCTTGCCAGCCCGCTCAG	CGTAGT	GCTGCCTCCCGTAGGAGT
50	GCCTTGCCAGCCCGCTCAG	CGTATC	GCTGCCTCCCGTAGGAGT

FIGURE 7D

REVERSE TAGGED ADAPTER PRIMER

Primer Pair	ADAPTER B	TAG	Reverse 16S rRNAPrimer
51	GCCTTGCCAGCCCGCTCAG	CGTCGT	GCTGCCTCCCGTAGGAGT
52	GCCTTGCCAGCCCGCTCAG	CGTCTC	GCTGCCTCCCGTAGGAGT
53	GCCTTGCCAGCCCGCTCAG	CGTGCA	GCTGCCTCCCGTAGGAGT
54	GCCTTGCCAGCCCGCTCAG	CGTGTC	GCTGCCTCCCGTAGGAGT
55	GCCTTGCCAGCCCGCTCAG	TACAGT	GCTGCCTCCCGTAGGAGT
56	GCCTTGCCAGCCCGCTCAG	TACATC	GCTGCCTCCCGTAGGAGT
57	GCCTTGCCAGCCCGCTCAG	TACGTC	GCTGCCTCCCGTAGGAGT
58	GCCTTGCCAGCCCGCTCAG	TACTGT	GCTGCCTCCCGTAGGAGT
59	GCCTTGCCAGCCCGCTCAG	TAGAGT	GCTGCCTCCCGTAGGAGT
60	GCCTTGCCAGCCCGCTCAG	TAGATC	GCTGCCTCCCGTAGGAGT
61	GCCTTGCCAGCCCGCTCAG	TAGCGT	GCTGCCTCCCGTAGGAGT
62	GCCTTGCCAGCCCGCTCAG	TAGCTC	GCTGCCTCCCGTAGGAGT
63	GCCTTGCCAGCCCGCTCAG	TAGTGT	GCTGCCTCCCGTAGGAGT
64	GCCTTGCCAGCCCGCTCAG	TATAGT	GCTGCCTCCCGTAGGAGT
65	GCCTTGCCAGCCCGCTCAG	TATATC	GCTGCCTCCCGTAGGAGT
66	GCCTTGCCAGCCCGCTCAG	TATCGT	GCTGCCTCCCGTAGGAGT
67	GCCTTGCCAGCCCGCTCAG	TATCTC	GCTGCCTCCCGTAGGAGT
68	GCCTTGCCAGCCCGCTCAG	TATGTC	GCTGCCTCCCGTAGGAGT
69	GCCTTGCCAGCCCGCTCAG	TCACGT	GCTGCCTCCCGTAGGAGT
70	GCCTTGCCAGCCCGCTCAG	TCACTC	GCTGCCTCCCGTAGGAGT
71	GCCTTGCCAGCCCGCTCAG	TCAGTC	GCTGCCTCCCGTAGGAGT
72	GCCTTGCCAGCCCGCTCAG	TCATGT	GCTGCCTCCCGTAGGAGT
73	GCCTTGCCAGCCCGCTCAG	TCGAGT	GCTGCCTCCCGTAGGAGT
74	GCCTTGCCAGCCCGCTCAG	TCGATC	GCTGCCTCCCGTAGGAGT
75	GCCTTGCCAGCCCGCTCAG	TCGCGT	GCTGCCTCCCGTAGGAGT
76	GCCTTGCCAGCCCGCTCAG	TCGCTC	GCTGCCTCCCGTAGGAGT
77	GCCTTGCCAGCCCGCTCAG	TCGTGT	GCTGCCTCCCGTAGGAGT
78	GCCTTGCCAGCCCGCTCAG	TCTAGT	GCTGCCTCCCGTAGGAGT
79	GCCTTGCCAGCCCGCTCAG	TCTATC	GCTGCCTCCCGTAGGAGT
80	GCCTTGCCAGCCCGCTCAG	TCTCGT	GCTGCCTCCCGTAGGAGT
81	GCCTTGCCAGCCCGCTCAG	TCTCTC	GCTGCCTCCCGTAGGAGT
82	GCCTTGCCAGCCCGCTCAG	TCTGTC	GCTGCCTCCCGTAGGAGT
83	GCCTTGCCAGCCCGCTCAG	AGAGCA	GCTGCCTCCCGTAGGAGT
84	GCCTTGCCAGCCCGCTCAG	AGATCA	GCTGCCTCCCGTAGGAGT
85	GCCTTGCCAGCCCGCTCAG	AGCACA	GCTGCCTCCCGTAGGAGT
86	GCCTTGCCAGCCCGCTCAG	AGCGCA	GCTGCCTCCCGTAGGAGT
87	GCCTTGCCAGCCCGCTCAG	AGCTCA	GCTGCCTCCCGTAGGAGT
88	GCCTTGCCAGCCCGCTCAG	AGTACA	GCTGCCTCCCGTAGGAGT
89	GCCTTGCCAGCCCGCTCAG	AGTGCA	GCTGCCTCCCGTAGGAGT
90	GCCTTGCCAGCCCGCTCAG	ATAGCA	GCTGCCTCCCGTAGGAGT
91	GCCTTGCCAGCCCGCTCAG	ATATCA	GCTGCCTCCCGTAGGAGT
92	GCCTTGCCAGCCCGCTCAG	ATCACA	GCTGCCTCCCGTAGGAGT
93	GCCTTGCCAGCCCGCTCAG	ATCGCA	GCTGCCTCCCGTAGGAGT
94	GCCTTGCCAGCCCGCTCAG	ATCTCA	GCTGCCTCCCGTAGGAGT
95	GCCTTGCCAGCCCGCTCAG	ATGACA	GCTGCCTCCCGTAGGAGT
96	GCCTTGCCAGCCCGCTCAG	ATGTCA	GCTGCCTCCCGTAGGAGT

Transcriptome **sequencing** with the **Genome** Sequencer FLX system

T Jarvie, T Harkins - Nature Methods, 2008 - nature.com

... Information on how to prepare small RNAs for 454 **Sequencing** analysis is available (Jarvie, T. & Harkins ... The **Genome** Sequencer FLX supports a number of formats, allowing users to customize the number of ... Multiplexing of samples is also supported by **Multiplex Identifier** tags. ...

Cited by 25 Related articles All 5 versions Cite Save

De novo 454 **sequencing** of barcoded BAC pools for comprehensive gene survey and **genome** analysis in the complex **genome** of barley

B Steuernagel, S Taudien... - BMC ..., 2009 - bmcgenomics.biomedcentral.com

... Due to the lack of a reference **genome** this BAC pool **sequencing** approach is ... short sequence tags that can be introduced either before the 454 **sequencing** library preparation [27] or by ligation of individual adaptors ("MID" = **Multiplex Identifier**", Roche Diagnostics ...

Cited by 69 Related articles All 14 versions Cite Save More

Digital PCR provides sensitive and absolute calibration for high throughput **sequencing**

RA White, PC Blainey, HC Fan... - BMC ..., 2009 - bmcgenomics.biomedcentral.com

... These results indicate that better than one-fold coverage of the ~4.7 Mbp K12 **genome** can be obtained from less than 1 ng input DNA. Detailed information about these **sequencing** results is presented in Table 3. Table 3 454 FLX trace library sequence results. Sample ID. ...

Cited by 149 Related articles All 39 versions Cite Save More

High-resolution, high-throughput HLA genotyping by next-generation **sequencing**

G Bentley, P Higuchi, B Hoglund, D Goodridge... - Tissue ..., 2009 - Wiley Online Library

... which allows the 454-**genome** sequencer software to differentiate HLA amplicon derived sequences from internal control sequences. We added 4-base **multiplex identifier** (MID) sequences (18) immediately following the library key tag to allow for multiplexed **sequencing** of HLA ...

Cited by 136 Related articles All 12 versions Cite Save

Phylotyping and functional analysis of two ancient human microbiomes

RY Tito, S Macmil, G Wiley, F Najar, L Cleeland, C Qu... - PLoS ..., 2008 - journals.plos.org

... Our matches represent sequences that are random areas of the **genome**; they are unlikely to be as robust as matches derived from ... False matches during shotgun **sequencing** are a concern [26]. ... Library formation employed **Multiplex Identifier** Kits (Roche Applied Science). ...

Cited by 41 Related articles All 20 versions Cite Save More

Rapid high-throughput human leukocyte antigen typing by massively parallel pyrosequencing for high-resolution allele identification

C Gabriel, M Danzer, C Hackl, G Kopal, P Rufnagl... - Human Immunology, 2009 - Elsevier

... the readout data per flow, which was then used by a **Genome** Sequencer-specific ... 2.0) of the GS-FLX amplicon variant analyzer (AVA) software supporting **multiplex identifier** sequence tags. In general, the AVA software maps the amplicon **sequencing** reads against a reference ...

Cited by 68 Related articles All 6 versions Cite Save

System and method for identification of individual samples from a multiplex mixture

MS Braverman, JF Simons, M Srinivasan ... - US Patent App. 12/ ..., 2008 - Google Patents

... a unique identifier (UID) element, which is sometimes also referred to as a **multiplex identifier** (MID), with ... refer to a nucleic acid molecule that is the subject of a **sequencing** reaction from ... refers to a collection of molecules derived from and/or representing an entire **genome** (ie all ...

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00792

Supplemental Information

C Vilek, BJ Kronschnabel, A Jeannard, G Blanc... - 2002 - researchgate.net

... 22 Page 2. Page2 of69 Selected DNA fragments were ligated to a Rapid Library **Multiplex Identifier** (MID) 23 adaptor designed for GS FLX Titanium chemistry. ... **genome** from duplicate **sequencing** runs; however, some regions had approximately100X 62 ...

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Improving Alfalfa as a Biofuel Feedstock

MS Genes - dl.sciencesocieties.org

... Using Next-Generation **Sequencing** in Amaranth Plant **Genome** 260–270 Although mostly underutilized ... from several individuals, and does not require a priori **genome** sequence information. ... with each sample labeled inde- pendently using a unique **multiplex identifier** barcode. ...

Cite Save

A NEW STRATEGY TO IDENTIFY THE DISEASE CAUSING MUTATION FOR NEURONAL CEROID LIPOFUSCINOSIS IN SOUTH HAMSPHIRE SHEEP

IFM Ismail, JAL Cavanagh, NL Mitchell... - Proc. Assoc. Advmt. ..., 2009 - aaabg.org

... The resulting products will be individually **multiplex identifier** (MID) tagged and 454 sequenced. CONCLUSION Whole **genome sequencing** has not been completed for the sheep and the publically available sequence information for our region of interest had large gaps. ...

Related articles All 6 versions Cite Save More

SNP discovery via genomic reduction, barcoding, and 454-pyrosequencing in amaranth

PJ Maughan, SM Yourstone, EN Jellen ... - ... plant **genome**, 2009 - dl.sciencesocieties.org

... based on restriction site conservation (GR-RSC) with each sample labeled independently using a unique **multiplex identifier** (MID) barcode ... bead separation and size selection via gel electrophoresis, followed by >10-fold **sequencing** coverage of the remaining **genome** via 454 ...

Cited by 67 Related articles All 6 versions Cite Save

Major histocompatibility complex genotyping with massively parallel pyrosequencing

RW Wiseman, JA Karl, BN Bimber, CE O'leary... - Nature medicine, 2009 - nature.com

... Chinese-origin rhesus macaques in a single pilot run on a **Genome** Sequencer FLX ... products from 12 macaques that were distinguished by 10-bp **multiplex identifier** (MID) tags ... detected five sequences not previously observed by cloning and Sanger **sequencing** (transcript levels ...

Cited by 92 Related articles All 15 versions Cite Save

Shifting the genomic gold standard for the prokaryotic species definition

M Richter, R Rosselló-Móra - Proceedings of the National ..., 2009 - National Acad Sciences

... For this, each **genome** was tagged with a different **multiplex identifier** for 454 libraries (Roche Applied ... almost duplicated until the bias is solved by the company producing the **sequencing** kits ... strain K12 that accounted for ≈3,158 reads covering ≈0.7 Mb (≈15% of the **genome**). ...

Cited by 869 Related articles All 13 versions Cite Save

CO dehydrogenase genes found in metagenomic fosmid clones from the deep Mediterranean Sea

AB Martín-Cuadrado, R Ghai, A Gonzaga... - Applied and ..., 2009 - Am Soc Microbiol

... approximately 7.2 Mb of DNA sequence (ie, roughly two prokaryotic **genome** equivalents ... For the **sequencing** process, the concentration of each DNA fosmid was measured using ... GATC, Constance, Germany), tagging each one individually using a **multiplex identifier** adaptor that ...

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00793

Identification of novel MHC class I sequences in pig-tailed macaques by amplicon pyrosequencing and full-length cDNA cloning and **sequencing**

CE O'Leary, RW Wiseman, JA Karl, BN Bimber ... - Immunogenetics, 2009 - Springer

... 3'), which binds a conserved region of exon 3. Forward and reverse PCR primer pairs contained one of 12 **multiplex identifier** (MID) tags ... steps were performed following the manufacturer's GS FLX protocols at the University of Illinois—Urbana Champaign **Sequencing** Center. ...

Cited by 28 Related articles All 13 versions Cite Save

Cost-effective sequence-based nonhuman primate MHC class I genotyping from RNA

JA Karl, RW Wiseman, DH O'Connor - Methods, 2009 - Elsevier

... based genotyping that amplifies full-length NHP class I cDNAs that are molecularly barcoded with **multiplex identifier** (MID) tags. ... Finally, this method can also be adapted for use on next-generation **sequencing** platforms such as the Roche/454 **Genome** Sequencer FLX ...

Cited by 10 Related articles All 9 versions Cite Save

System and method for improved processing of nucleic acids for production of sequencable libraries

GC Ferreri, JF Simons, MT Roman ... - US Patent App. 12/ ..., 2009 - Google Patents

... data for each amplified member that comprises a sequence composition for the **multiplex identifier** element; and ... from and/or representing an entire **genome** (ie all regions of a **genome**) of an ... term "keypass" or "keypass well" as used herein generally refers to the **sequencing** of a ...

Cited by 2 Related articles All 2 versions Cite Save

System and method for detection of HIV integrase variants

BB Simen, EPS John - US Patent App. 12/592,243, 2009 - Google Patents

... from and/or representing an entire **genome** (ie all regions of a **genome**) of an ... functions including, but not limited to, primer sequences for amplification and/or **sequencing** methods, quality control elements, unique identifiers (also referred to as a **multiplex identifier** or "MID ...

Related articles All 2 versions Cite Save

Mycobacteriophages BPs, Angel and Halo: comparative genomics reveals a novel class of ultra-small mobile genetic elements

T Sampson, GW Broussard, LJ Marinelli, ... - ..., 2009 - mic.microbiologyresearch.org

... and annealed to primer-coated beads; the 'A' adaptor contained a 10 bp **Multiplex Identifier** (MID) tag ... per read, providing a total of 1 636 726 bases of raw **sequencing** information. ... one large contig with consensus quality values of 60 or greater and average **genome** coverage of ...

Cited by 47 Related articles All 13 versions Cite Save

Best Abstracts

A Amir, L Dorscgna, M van Loenen, M Kester... - 2009 - Wiley Online Library

... Gene **sequencing** showed the presence of a new type of mutation in the TAP1 gene. ... Page 3. O6 Analysis of the KIR in the CEPH human **genome** diversity panel reveals evidence of different evolutionary histories for the centromeric and telomeric portions of the gene cluster Jill A ...

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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L23	21	(brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "10X GENOMICS".as. or "COMPASS GENETICS".as. or "population genetics".as.) and (nucleic or dna or polynucleotide)same (adaptor or adapter or linker) same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:32
L24	8	23 and sequencing.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:32
L25	1	24 and (single or individual or one) near3 cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:34
L26	134	(brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "10X GENOMICS".as. or "COMPASS GENETICS".as. or "population genetics".as.) and (single or individual or one) near3 cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:35
L27	3	26 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)same sequence same (identif$4 or detect$3 or determin$3 or distinguish$3) same cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:36
L28	29	"9410150"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 18:04
L29	8	"9410149".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 18:05

L30	3	"8563274".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 18:06
L31	2	"8679756".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 18:08

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EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	29794	brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "10X GENOMICS".as. or "COMPASS GENETICS".as. or "population genetics".as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 10:50
S2	134	S1 and (single or individual or one) near3 cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 10:51
S3	536	S1 and (dna or nucleic or polynucleotide) same (pcr or amplif$7)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 10:54
S4	18	S3 and (adapt$3 or linker) near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 10:56
S5	4	S4 and sequencing same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 10:58
S6	139392	(single or individual or one) near3 cell same (dna or nucleic or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:01
S7	3492	S6 and (adapt$3 or linker) near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:02
S8	872	S7 and sequencing same (mid or	US-PGPUB;	OR	ON	2017/01/23

		"multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence	USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			11:02
S9	88	S8 and (single or individual or one) near3 cell near5 first	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:03
S10	84	S8 and (single or individual or one) near3 cell near5 second	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:03
S11	66	S10 and first near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:05
S12	47	S11 and second near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:05
S13	43263	"first cell" and "second cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:15
S14	1761	S13 and (dna or nucleic or polynucleotide) same "first cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:16
S15	1549	S14 and (dna or nucleic or polynucleotide) same "second cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:17
S16	143	S15 and (adapt$3 or linker) same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT;	OR	ON	2017/01/23 11:18

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			IBM_TDB			
S17	27	S16 and second near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier) near5 first	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:19
S18	35	S16 and sequencing same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:20
S19	870	(single or individual or one) near3 cell near5 isolat$4 near5 first	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:29
S20	315	S19 and (single or individual or one) near3 cell near5 isolat$4 near5 second	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:30
S21	120	S20 and (dna or nucleic or polynucleotide) near5 cell	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:31
S22	25	S21 and (adaptor or adapter or linker) same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:31
S23	12	S21 and sequencing same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:34
S24	540	sequencing same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence same cell near5 (type or individual$3 or single)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:36
S25	381	S24 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3	US-PGPUB; USPAT; USOCR; FPRS; EPO;	OR	ON	2017/01/23 11:37

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		sequence same (identif$4 or detect$3 or dtermin$3) same cell near5 (type or individual$3 or single)	JPO; DERWENT; IBM_TDB			
S26	67	S25 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence near5 (identif$4 or detect$3 or dtermin$3) same cell adj (type or individual$3 or single)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:38
S27	2799	"first cell" same (nucleic or dna or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:47
S28	1549	S27 and "second cell" same (nucleic or dna or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:48
S29	1	S28 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence near5 (identif$4 or detect$3 or determin$3) near5 "first cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:49
S30	8	S27 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence same (identif$4 or detect$3 or determin$3) near5 "first cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:51
S36	2321	(mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near5 sequence near5 (identif$4 or detect$3 or determin$3 or distinguish$3) near5 (cell or sample)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:59
S37	46	S36 and (append$3 or ligat$3) near5 (adpator or adapter or linker) near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier) same (dna or nucleic or polynucleotide) near5 (cell or sample)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 12:02

1/23/2017 4:58:45 PM
C:\Users\nbhat1\Documents\EAST\Workspaces\14792094-1-23-17.wsp

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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14792094	BRENNER ET AL.
	Examiner	Art Unit
	NARAYAN BHAT	1634

CPC- SEARCHED		
Symbol	Date	Examiner
C12Q 1/68, C12Q 1/6874, C07H 21/02 and C40B 30/04	1/6/16	Bhat N.
Searched uisng key words in different CPC classes. See the search history in EAST.	1/6/16	Bhat N.

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Reviewed the copending applications for the inventors in PLAM and copending claims for potential ODP issues.	1/6/16	Bhat N.
Reviewed the prosecution histories of copending applications resulting in the issuance of patents.	1/6/16	Bhat N.
Reviewed the cited arts in the IDS of 10/9/15.	1/6/16	Bhat N.
searched for the topics in EAST, Pubmed and Google scholar. Search histories are enclosed.	1/6/16	Bhat N.
Updated the search for the topics in EAST. Search history is enclosed.	7/17/16	Bhat N.
C12Q 1/68, C12Q 1/6874, C07H 21/02 and C40B 30/04	1/6/16	Bhat N.
Updated the search for the topics in EAST, Pubmed and Google scholar. Search histories are enclosed.		

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

	/NARAYAN BHAT/ Primary Examiner.Art Unit 1634

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UNITED STATES PATENT AND TRADEMARK OFFICE

USPTO Automated Interview Request (AIR)

Feb 27 2017

This paper requesting to schedule and/or conduct an interview is appropriate because:

This submission is requested to be accepted as an authorization for this interview to communicate via the internet. Recognizing that Internet communications are not secure, I hereby authorize the USPTO to communicate with the undersigned concerning scheduling of the interview via video conference, instant messaging, or electronic mail, and to conduct the interview in accordance with office practice including video conferencing.

Name(s):
Gargi Talukder

S-signature:
/Gargi Talukder/

Registration Number:
61368

U.S. Application Number:
14792094

Confirmation Number:
9697

E-mail Address:
gargi.talukder@morganlewis.com

Phone Number:
4154421266

Proposed Time of Interview:
3-16-2017 3:00 PM ET

Prefered Interview Type:
Telephonic

I am the applicant or applicant's representative for this application.

uspto UNITED STATES PATENT AND TRADEMARK OFFICE
PALM-SILVER

UNITED STATES PATENT AND TRADEMARK OFFICE

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

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/792,094	07/06/2015	Sydney Brenner	104371-5016-US05	9697

43850 7590 03/21/2017
MORGAN, LEWIS & BOCKIUS LLP (SF)
One Market, Spear Street Tower, Suite 2800
San Francisco, CA 94105

EXAMINER
BHAT, NARAYAN KAMESHWAR

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
03/21/2017	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):

sfipdocketing@morganlewis.com
donald.mixon@morganlewis.com

Applicant-Initiated Interview Summary	**Application No.** 14/792,094	**Applicant(s)** BRENNER ET AL.	
	Examiner NARAYAN BHAT	**Art Unit** 1634	

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

(1) *Examiner: Bhat*. (3)_____.

(2) *Representative: Ms. Talukder*. (4)_____.

Date of Interview: *16 March 2017*.

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: *40*.

Identification of prior art discussed: *Braverman*.

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...)

In view of Automated Interview Request filed on 2/27/17, the interview was scheduled on 3/16/2017. The representative discussed that claim 40 languages are similar to the allowed claims of US 8,679,756 and 8,563,274 and further specifies the nucleic acid is from first and second isolated single cells and thus would be non-obvious over Braverman. The examiner informed that the allowed claims require "different segments of a first nucleic acid molecule of a contiguous region in genomic DNA, wherein said contiguous region in genomic DNA is represented by a plurality of nucleic acid molecules" which is different from what is claimed in claim 40 and there are no support for the phrase "isolated single cell" in the instant specification and the recitation of "first and second isolated cell" could be the same. The representative asked if the support for said phrase is identified in the instant specification, will the pending claims be allowable. The examiner informed that instant claim 40 would be obvious over the arts of the record. No specific claim languages were agreed upon 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

/NARAYAN BHAT/ Primary Examiner, Art Unit 1634	

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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.

Attachment: IMIS (2)

From:	PTO Automated Interview Request
To:	Bhat, Narayan
Cc:	PTO Automated Interview Request
Subject:	USPTO Automated Interview Request (AIR): Action Required - Received interview for US Application Number 14792094
Date:	Monday, February 27, 2017 2:37:33 PM
Importance:	High

NARAYAN BHAT,

This is an Automated Interview Request (AIR) made by Gargi Talukder for application number 14792094 for 3-16-2017 3:00 PM ET. The preferred interview type is: **Telephonic**. Please respond to this interview request within **1 business day**.

Please contact Gargi Talukder by calling 4154421266 or emailing gargi.talukder@morganlewis.com to set up an interview or deny if appropriate.

By receiving this AIR submission, the applicant has certified that:
This submission is requested to be accepted as an authorization for this interview to communicate via the internet. Recognizing that Internet communications are not secure, I hereby authorize the USPTO to communicate with the undersigned concerning scheduling of the interview via video conference, instant messaging, or electronic mail, and to conduct the interview in accordance with office practice including video conferencing.

If you're going to deny the interview, please contact your SPE to clear the tracking off of your dashboard.

If you need assistance please contact your SPE, an Interview Specialist from your TC or see Quick Reference Guide. Thank you.

Please do not reply to this message; it was sent from an unmonitored e-mail address.

From:	Talukder, Gargi
To:	Bhat, Narayan
Subject:	RE: In preparation for Interview on March 16, 2017 for USSN 14/792,094 (MLB Ref: 104371-5016-US05) 3.30 EST
Date:	Wednesday, March 15, 2017 4:48:03 PM

Examiner Bhat,

Yes, I will give you a call at 3:30 pm EST tomorrow.

Thanks,
Gargi

Gargi Talukder, J.D., Ph.D.
Morgan, Lewis & Bockius LLP
One Market, Spear Street Tower | San Francisco, CA 94105
Direct: +1.415.442.1266 | Main: +1.415.442.1000 | Fax: +1.415.442.1001
gargi.talukder@morganlewis.com | www.morganlewis.com
Assistant: Ronn R. Uchihara | +1.415.442.1715 | ronn.uchihara@morganlewis.com

From: Bhat, Narayan [mailto:Narayan.Bhat@USPTO.GOV]
Sent: Wednesday, March 15, 2017 1:36 PM
To: Talukder, Gargi
Subject: RE: In preparation for Interview on March 16, 2017 for USSN 14/792,094 (MLB Ref: 104371-5016-US05) 3.30 EST

Ms. Talukder,
Sorry for the mix up. I have an interview tomorrow at 2 pm. Can we have the interview tomorrow @ 3.30 EST instead at 3 pm ? Thank you very much for your cooperation.

Narayan K Bhat
Primary Examiner, AU 1634,
Remsen 3C09, TC 1600, USPTO, DOC, Alexandria, VA-22313
T: 571-272-5540
Fax: 571-273-5540

From: Talukder, Gargi [mailto:gargi.talukder@morganlewis.com]
Sent: Wednesday, March 15, 2017 4:29 PM
To: Bhat, Narayan <Narayan.Bhat@USPTO.GOV>
Subject: FW: In preparation for Interview on March 16, 2017 for USSN 14/792,094 (MLB Ref: 104371-5016-US05)

Dear Examiner Bhat,

Further to my voicemail, please see the forwarded e-mail below confirming that our interview is scheduled for tomorrow. Please let me know if tomorrow at 3 pm ET still works for you.

Thank you,
Gargi Talukder

Gargi Talukder, J.D., Ph.D.
Morgan, Lewis & Bockius LLP
One Market, Spear Street Tower | San Francisco, CA 94105
Direct: +1.415.442.1266 | Main: +1.415.442.1000 | Fax: +1.415.442.1001
gargi.talukder@morganlewis.com | www.morganlewis.com
Assistant: Ronn R. Uchihara | +1.415.442.1715 | ronn.uchihara@morganlewis.com

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From: Talukder, Gargi
Sent: Monday, February 27, 2017 5:46 PM
To: narayan.bhat@uspto.gov
Cc: Gloss, Sheila R.
Subject: In preparation for Interview on March 16, 2017 for USSN 14/792,094 (MLB Ref: 104371-5016-US05)

Dear Examiner Bhat,

Thank you for taking the time to speak with me today, and I look forward to our further discussion in a couple of weeks.

Per our discussion today, please refer to US Patent No. 8,679,756 (USSN 14/021,790) and US Patent No. 8,563,274 (USSN 13/859,450). These are the patents on which we modeled the claims for the current case.

Best regards,
Gargi Talukder

Gargi Talukder, J.D., Ph.D.
Morgan, Lewis & Bockius LLP
One Market, Spear Street Tower | San Francisco, CA 94105
Direct: +1.415.442.1266 | Main: +1.415.442.1000 | Fax: +1.415.442.1001
gargi.talukder@morganlewis.com | www.morganlewis.com
Assistant: Ronn R. Uchihara | +1.415.442.1715 | ronn.uchihara@morganlewis.com

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 9697
Sidney Brenner et al.	Examiner: Narayan Kameshwar Bhat
Application No.: 14/792,094	Art Unit: 1634
Filed: July 6, 2015	
For: COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID ARRANGEMENT	RESPONSE TO OFFICE ACTION

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

Commissioner:

In response to the Office Action mailed February 1, 2017, please enter the following amendments and remarks:

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

Remarks/Arguments begin on page **5** of this paper.

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Amendments to the Claims:

This listing of claims will replace all prior versions, and listings of claims in the application:

Listing of Claims:

1-39 (Cancelled)

40. (Currently amended) A method of analyzing nucleic acids, the method comprising:

(a) providing a first plurality of segments of nucleic acid molecules from a contiguous region of a genome from a first single isolated cell, wherein each of the first plurality of segments is appended to a first multiplex identifier (MID) sequence prior to or during an amplification reaction to form first appended segments;

(b) providing a second plurality of segments of nucleic acid molecules from a contiguous region of a genome from a second single isolated cell, wherein each of the second plurality of segments is appended to a second MID sequence prior to or during an amplification reaction to produce second appended segments, wherein the first single isolated cell is a different cell than the second single isolated cell, and wherein the first MID sequence and the second MID sequence are different from each other;

(c) sequencing the first appended segments and the second appended segments to provide a plurality of sequences,

wherein each sequence comprises (i) nucleotide sequence of at least a portion of a segment and (ii) an associated MID sequence, and wherein the MID sequences uniquely identify the segments with a single isolated cell.

41. (Previously presented) The method of claim 40, wherein the method further comprises amplifying the first appended segments and the second appended segments before the sequencing step (c).

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42. (Previously presented) The method of claim 40, wherein the nucleic acid molecules are selected from DNA and RNA.

43. (Previously presented) The method of claim 42, wherein the nucleic acid molecules comprise mRNA.

44. (Previously presented) The method of claim 40, wherein the MID sequences are appended to the segments through ligation.

45. (Previously presented) The method of claim 40, wherein the MID sequences are appended to the segments by performing PCR or linear amplification of the plurality of nucleic acids using an adapter sequence comprising the MID sequence and an amplification primer.

46. (Previously presented) The method of claim 40, wherein the sequences of the segments are attributed to different isolated cells based on the associated MID sequence.

47. (Previously presented) The method of claim 40, wherein the MID sequences further comprise a sequencing primer sequence.

48. (Previously presented) The method of claim 40, wherein the MID sequences are from 2 to 100 bases in length.

49. (Previously presented) The method of claim 40, wherein the segments of (a) and (b) are in the range of 100 to 5,000 bp in length.

50. (Previously presented) The method of claim 40, wherein the segments of (a) and (b) are 400 bp or less in length.

51. (Previously presented) The method of claim 40, wherein the method further comprises providing a third plurality of segments of nucleic acid molecules from a contiguous region of a genome from a third single isolated cell, wherein each of the third plurality of segments is appended to a third MID sequence to produce third

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appended segments, and the third MID sequence is different from the first MID sequence and the second MID sequence.

52. (Previously presented) The method of claim 40, wherein the contiguous region is greater than 2 kb in length.

53. (Currently amended) A method of analyzing a plurality of contiguous genomic sequences, comprising:

(a) providing a sample comprising the plurality of contiguous genomic sequences, wherein the plurality of contiguous genomic sequences are from a plurality of single isolated cells;

(b) tagging a plurality of fragments of the contiguous genomic sequences with a multiplex identifier (MID) sequence prior to or during an amplification reaction such that fragments of a first contiguous nucleic acid sequence share a ~~common~~ MID sequence that is different from ~~an~~ a MID sequence of fragments of a second contiguous nucleic acid sequence; and

(c) sequencing the plurality of fragments and MID sequences to identify fragments of the contiguous sequence.

54. (Previously presented) The method of claim 53, wherein the first contiguous nucleic acid sequence is from a first single isolated cell and the second contiguous nucleic acid sequence is from a second single isolated cell.

55. (Previously presented) The method of claim 53, wherein sequences of the fragments are attributed to different single isolated cells based on the associated MID sequence.

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Remarks/Arguments

Applicants have not dedicated or abandoned any unclaimed subject matter and have not acquiesced to any rejections made by the Patent Office. Applicants reserve the right to pursue prosecution of any presently excluded claim embodiments in future continuation and/or divisional applications.

Status of the Claims

Claims 40-55 are pending and under consideration in this application. Claims 40 and 53 have been amended, and these amendments are fully supported in the specification as filed.

For example, the amendment that the first and second MID sequences are appended "prior to or during an amplification reaction" is supported on pages 4-5 and 24-25 of the specification as filed.

The amendment clarifying that the first and second cells are different cells is supported for example on page 8, which states that "a nucleic acid sample may be a pool of polynucleotides derived from different sources, (e.g., polynucleotides derived from different individuals, different tissues *or cells*, or polynucleotides isolated at different times points)..." (emphasis added).

Applicants respectfully request entry of the claims as amended into the record.

Interview summary

Examiner Bhat and Applicants' representative, Gargi Talukder, (herein together referred to as "the Parties") participated in a telephonic interview on March 16, 2017. Applicants thank Examiner Bhat for his attention and time to this matter.

The Parties discussed the pending claims and the outstanding rejections in the present office action. No agreement was reached on the claims, and the Examiner suggested that a response to office action be filed with further amendments and arguments for consideration. The

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Examiner indicated he may need to conduct further searches depending on any new amendments to the claims.

Response to Office Action

Claim Objections

Claim 53 is objected to because of a typographical error to the recitation of "an MID sequence" in step 'b' lines 3 and 4. Claim 53 has been amended as suggested by the Examiner, thus overcoming this objection. Applicants respectfully request that this rejection be withdrawn.

Claim Rejections – 35 USC § 112-first paragraph – written description

Claims 40-55 are rejected under 35 U.S.C. § 112(a) or 35 U.S.C. § 112 (pre-AIA), first paragraph, as allegedly failing to comply with the written description requirement. Applicants respectfully disagree.

The Office asserts on page 7 of the current Office Action that the claim elements "first single isolated cell," "second single isolated cell," and "a common MID sequence" constitute new matter.

Applicants respectfully remind the Office that word-for-word support for every element of a claim is not the standard for fulfillment of the written description requirement. Rather, "If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met. See, e.g., *Vas-Cath,* 935 F.2d at 1563, 19 USPQ2d at 1116; *Martin v. Johnson,* 454 F.2d 746, 751, 172 USPQ 391, 395 (CCPA 1972) (stating "the description need not be in *ipsis verbis* [i.e., "in the same words"] to be sufficient")." *MPEP 2163.* Applicants submit that the specification as filed provides an adequate description of all of the elements of the present claims, including the elements identified in the present Office Action.

Taking the "first single isolated cell" and "second single isolated cell" elements first, Applicants note that the specification explicitly states that samples can include polynucleotides

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from different sources, including "different tissues or cells." *See page 8, line 5 of the present specification.* In addition, the specification further provides that the nucleic acid sample can be from a single source, such as a cell, or from of a plurality of cells where by plurality is meant "two or more." *See page 19, first full paragraph of the specification as filed.* As such, the skilled artisan would readily understand that the specification as filed supports methods in which the polynucleotides to be analyzed can be sourced from two different cells, because the specification states that two or more cells could be the source of the nucleic acid sample. As will be appreciated, an adequate written description of the invention "may be shown by any description of sufficient, relevant, identifying characteristics so long as a person skilled in the art would recognize that the inventor had possession of the claimed invention." *MPEP §2163.* Applicants submit that the above excerpts from the present specification provides sufficient identifying characteristics of the claimed invention to fulfill the written description requirement.

For the element of a "common MID sequence" Applicants have, solely to further prosecution of this application and without conceding to the propriety of this rejection, amended claim 53 to remove the term "common," thus clarifying that the claimed invention involves polynucleotides where fragments of a first contiguous nucleic acid sequence share an MID sequence that is different from an MID sequence of fragments of a second contiguous nucleic acid sequence. As such, the written description rejection as it pertains to this element of the claims is rendered moot.

For at least the foregoing reasons, Applicants respectfully request that all rejections under § 112 for lack of written description be withdrawn.

Claim Rejections – 35 USC § 112-second paragraph

Claims 53-55 are rejected under 35 U.S.C. § 112(b) or 35 U.S.C. § 112 (pre-AIA), second paragraph, as being indefinite for allegedly 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. Applicants respectfully disagree.

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The Office Action states on page 9 that claim 53 is indefinite because "in step 'b' it is unclear "how sharing sequences is different." Applicants note that the claim recites that fragments of a first contiguous nucleic acid sequence share a MID sequence that is different from *a MID sequence of fragments of a second contiguous nucleic acid sequence*. In other words, the fragments from the first contiguous nucleic acid sequence are tagged with the same MID sequence, and that MID sequence is different than the MID sequence that tags the fragments of the second nucleic acid sequence. Applicants respectfully submit that the claim is clear as currently pending and request that this rejection be withdrawn.

Claim Rejections – 35 USC § 103

Claims 40-55 are rejected under 35 U.S.C. § 103 as being allegedly anticipated by Braverman et al., US 2009/0105959 ("Braverman") in view of Drmanac, US 2007/0072208 ("Drmanac"). Applicants respectfully traverse these rejections.

Applicants submit that the skilled artisan would not have found it obvious to combine the teachings of Braverman and Drmanac to practice the claimed invention because (i) Braverman teaches away from elements of the claimed invention, (ii) the skilled artisan would have no reasonable expectation of success in practicing the claimed invention by combining the teachings of Braverman and Drmanac, and (iii) the combined teachings of Braverman and Drmanac fail to disclose all elements of the claimed invention.

As discussed in *KSR v. Teleflex*, "when the prior art teaches away from combining certain known elements, discovery of a successful means of combining them is more likely to be nonobvious." *KSR Int'l Co. v. Teleflex, Inc., 550 U.S. 398 (2007) citing United States v. Adams, 383 U.S. 39, 51-52 (1966)*. In addition, as described in MPEP § 2141.02, a prior art reference must be considered "in its entirety, i.e., as a whole, including portions that would lead away from the claimed invention." *W.L. Gore & Assoc., Inc. v. Garlock, Inc.*, 721 F.2d 1540, 220 USPQ 303 (Fed. Cir. 1983), *cert. denied,* 469 U.S. 851 (1984).

When considered as a whole, Braverman is directed to associating nucleic acids with a "UID element" that is "resistant to introduced error in sequence data." *Braverman, paragraph*

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[0028]. Toward its purpose of producing nucleic acids tagged with UID's that are resistant to introduced error, Braverman teaches that methods of attaching the UID molecules after amplification provide distinct advantages: "By coupling the UID elements to the adapted template molecules post-amplification, errors introduced by the amplification method are avoided." *Braverman, paragraph [0084]*. The present claims are directed to methods in which an MID are appended prior to or during an amplification reaction. According to Braverman, any such tags would be prone to errors, and as such, Braverman teaches away from the claimed invention. The skilled artisan would not find it obvious to combine these teachings in Braverman with those in Drmanac to practice the claimed invention.

Furthermore, the skilled artisan would have no reasonable expectation of success in practicing the claimed invention by combining the teachings of Braverman with those of Drmanac. Drmanac describes a method that relies upon a dilution process that results in forming aliquots containing mixtures of non-overlapping fragments. In fact, the title of Drmanac is "Nucleic acid analysis by random mixtures of non-overlapping fragments." The methods in Drmanac rely on the dilution process to order sequence information. For example, Drmanac states that "When the fragments in the final tier are sequenced, overlapping sequence regions of fragments in different aliquots are used to register the different fragments so that non-overlapping regions are ordered." *Drmanac, paragraph [0015]*. As further explained in Drmanac, an overriding feature of the process in Drmanac is that different aliquots at most contain one molecule of a particular sequence of interest: "A common feature in all applications of this invention (genome sequencing, SNP or other marker mapping or cDNA analysis) is aliquoting nucleic acid sample such that sequences of predetermined type of relatedness (overlapped fragment, fragments with high similarity, homologous chromosomes, messengers transcribed from the same gene) occur mostly once e.g. as a single molecule per aliquot." *Drmanac, paragraph [0017]*. The skilled artisan would have no reason to use the teachings of Drmanac in combination with those of Braverman to practice the claimed invention, because the purpose of Drmanac is to divide out the nucleic acids from a source to sub-haploid levels, whereas the presently claimed invention would result in nucleic acids from a first cell to be tagged with an MID that is different from the MID of nucleic acids form a second cell. In other words, the sequencing step of the presently claimed methods would necessarily involve

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sequencing overlapping segments from the original isolated cell. As such, the skilled artisan would have no expectation of success in practicing the claimed invention based on the combined teachings of Drmanac and Braverman, because Drmanac is directed to a method that is not in keeping with the presently claimed invention.

Applicants note that the Office has cited to Drmanac to provide the teaching that the samples can be from two isolated cells. However, as discussed above, in supporting a rejection for obviousness, a prior art reference must be considered "in its entirety, i.e., as a whole, including portions that would lead away from the claimed invention." *W.L. Gore & Assoc., Inc. v. Garlock, Inc.*, 721 F.2d 1540, 220 USPQ 303 (Fed. Cir. 1983), *cert. denied,* 469 U.S. 851 (1984). *MPEP § 2141.02.* When taken as a whole, Drmanac does not support a rejection for obviousness.

Applicants further submit that the combined teachings of Braverman and Drmanac fail to disclose or suggest elements of the claims, including a method in which each of a first plurality of segments from a first isolated cell is appended to a first multiplex identifier (MID) and each of a second plurality of segments from a second isolated cell are appended to a second MID, as is recited in independent claim 40. In addition, the combined teachings of Braverman and Drmanac fail to provide a method in which a plurality of fragments of contiguous genomic sequences are tagged with a MID sequence such that fragments of a first contiguous nucleic acid sequence share a common MID sequence that is different from an MID sequence of fragments of a second contiguous nucleic acid sequence, as is recited in independent claim 53. Furthermore, the combined teachings of Braverman and Drmanac fails to disclose methods in which segments appended to or tagged with MIDs can be attributed to their single cell of origin.

For at least the foregoing reasons, Applicants respectfully request that all rejections under § 103 be withdrawn.

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Claim Rejections – Double Patenting

Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-12 of U.S. Patent No. 8,679,756 in view of Drmanac (US 2007/0072208).

For the reasons recited above, Applicants respectfully submit the presently claimed invention does not support a rejection on the ground of nonstatutory double patenting, as the present claims are not obvious in light of U.S. Patent No. 8,679,756 in view of Drmanac. Applicants respectfully request that this rejection be withdrawn.

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CONCLUSION

All the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn.

Applicants believe that a full and complete reply has been made to the outstanding Office Action, and as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided below.

Except for issue fees payable under 37 C.F.R. § 1.18, the Commissioner is hereby authorized by this paper to charge any necessary fees during the entire pendency of this application including fees due under 37 C.F.R. §§ 1.16 and 1.17, which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-0310 (Order No.: 104371-5016-US05).

Prompt and favorable consideration of this Amendment and Response is respectfully requested.

Respectfully submitted,
MORGAN, LEWIS & BOCKIUS LLP

Dated: May 1, 2017 By: Gargi Talukder
Gargi Talukder, Reg. No. 61,368

Customer No.: 43850
Morgan, Lewis & Bockius LLP
One Market, Spear Street Tower
San Francisco, CA 94105
Telephone: (415) 442-1000
Facsimile: (415) 442-1001

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Electronic Acknowledgement Receipt	
EFS ID:	29077048
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	43850
Filer:	Gargi Talukder/Sheila Gloss
Filer Authorized By:	Gargi Talukder
Attorney Docket Number:	104371-5016-US05
Receipt Date:	01-MAY-2017
Filing Date:	06-JUL-2015
Time Stamp:	14:20:00
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		104371_5016_US05_RSP.pdf	578756 2f64cca68f47177fdc921d9212ca436981866 7ca1	yes	12

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
	Claims	2	4
	Applicant Arguments/Remarks Made in an Amendment	5	12
Warnings:			
Information:			
	Total Files Size (in bytes):	578756	

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

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (09-11)
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/792,094	Filing Date 07/06/2015	☐ 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), (l), 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 05/01/2017

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 16	Minus	** 20	= 0	x $40 =	0
Independent (37 CFR 1.16(h))	* 2	Minus	***3	= 0	x $210 =	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	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.

LIE
ROBERT TALBERT

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/792,094	07/06/2015	Sydney Brenner	104371-5016-US05	9697

43850 7590 05/31/2017
MORGAN, LEWIS & BOCKIUS LLP (SF)
One Market, Spear Street Tower, Suite 2800
San Francisco, CA 94105

EXAMINER
BHAT, NARAYAN KAMESHWAR

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
05/31/2017	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):

sfipdocketing@morganlewis.com
donald.mixon@morganlewis.com

PTOL-90A (Rev. 04/07)

Office Action Summary	Application No. 14/792,094	Applicant(s) BRENNER ET AL.	
	Examiner NARAYAN BHAT	Art Unit 1634	AIA (First Inventor to File) Status No

-- 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 5/01/2017.

☐ 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) 40-55 is/are pending in the application.

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

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) 40-55 is/are rejected.

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

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

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

Application Papers

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

11)☒ The drawing(s) filed on 7/6/2015 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: _____.

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Final action

1. The present application is being examined under the pre-AIA first to invent provisions. This office action is in response to the papers filed on 5/1/2017. The amendments to independent claims 40 and 53 changing the scope of previously rejected said claims necessitated the new grounds of rejections as set forth in this office action. ***Accordingly this office action has been made final.***

Track1 Request

2. Track1 request filed by the applicant on December 22, 2016 has been granted by the office on January 10, 2017. Therefore instant application has been examined on a priority basis. To maintain its status applicant is required to follow the requirements listed in the Track1 grant document.

3. The present application is being examined under the pre-AIA first to invent provisions. The instant application is a continuation of 14/172,694, now U.S. Patent 9,102,980, which is a continuation of 14/021,790, now U.S. Patent 8,679,756, which is a continuation of 13/859,450, now U.S. Patent 8,563,274, which is a continuation of 13/622,872, now abandoned, which is a continuation of 13/387,343, now U.S. Patent 8,298,767, which is a national stage entry of PCT/IB2010/002243, claiming priority from provisional application 61288792, filed on 12/21/2009 and from provisional application 61235595, filed on 08/20/2009.

Claim Status

4. In the claim listing of 5/1/2017 claims 40-55 are pending in this application and are under prosecution. Claims 40 and 53 are amended. Claims 1-39 are canceled. Claims have been reviewed and entered. The claim amendments have been reviewed and entered. No new matter has been introduced by the claim amendments as applicant has identified the support in the instant specification (Remarks, pg. 5).

5. The interview summary filed by the applicant has been acknowledged by the examiner (Remarks, pg. 5)

Withdrawn Objection, Rejections and Response to the Remarks

6. The previous objection of claim 53 has been withdrawn in view of fixing the minor informality in said claim (Remarks, pg. 6).

7. The previous new matter rejection of claims 40-55 under 35 USC pre-AIA first paragraph has been withdrawn in view of claim amendments and clarifying on the record that the instant specification at the time of filing has the support for the first and the second isolated single cell (Remarks, pgs. 6 and 7).

8. The previous rejection of claims 53-55 under 35 USC pre-AIA second paragraph has been withdrawn in view of claim amendments (Remarks, pgs. 7 and 8).

9. The previous rejection of claims 40-55 under 35 USC 103(a) as being unpatentable over Braverman in view of Drmanac has been withdrawn in view of the claim amendments, persuasive arguments made by the applicant that Braverman does

not teach the step of appending MID sequences prior to or during an amplification reaction and solely to expedite the prosecution (Remarks, pgs. 8-10).

10. The previous ODP rejections on the record have been withdrawn necessitated by the claim amendments. The arguments regarding ODP rejection of instant claims 40-55 over claims 1-12 of '756 patent in view of Drmanac have been fully considered (Remarks, pg. 11). They are moot in view of withdrawn ODP rejections necessitated by the claim amendments.

However, upon further consideration new grounds of rejections are set forth in this office action necessitated by the claim amendments.

Claim Rejections - 35 USC § 103

11. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

12. Claims 40-55 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Gillevet (WO 2008/061193, cited in the previous office action) in view Wangh et al (US 2003/0022231 published Jan 30, 2003).

Gillevet and Wangh teach a method for analyzing nucleic acid from plurality of samples and therefore are analogous arts. Gillevet teaches the steps of claims 40 and 53 including analyzing the nucleic acid from a plurality of nucleic acid template from multiple samples and sequencing the segments of the nucleic acid template (i.e., contiguous region of the genome) from each sample and identifying the nucleic acid segments from each sample by a tag sequence (i.e., a multiplex identifier) specific to

each of sample. Gillevet does not specifically teach that the nucleic acid segments from single isolated cell from each sample. However, the concept of using the nucleic acid from single isolated cell from each sample was known in the art at the time the claimed invention was made as taught by Wangh, which is very useful for a variety of uses, including research, forensic science and diagnostic applications least two isolated single cells.

Regarding claim 40, Gillevet teaches a multiplex method for the quantitative determination of polynucleotides in two or more samples, comprising: hybridizing a first primer to polynucleotides in a first sample, said first primer comprising a first tag sequence (i.e., a first multiplex identifier (MID)) and a first probe sequence specific for a first target sequence, wherein said first target sequence is 3' to a variable genetic region; elongating primer templates formed thereby to form a first population of tagged polynucleotides comprising: said first primer including said first tag sequence (i.e., first MID sequence); and sequences of said variable genetic region (encompasses the step of appending first MID); hybridizing a second primer to polynucleotides in a second sample, said second primer comprising a second tag sequence (i.e., a second MID) and a second probe sequence specific for a second target sequence, wherein said second target sequence is 3' to the same variable genetic region as said first target sequence, wherein further said second probe sequence may be the same as or different from said first probe sequence; elongating primer templates formed thereby to form a second population of tagged polynucleotides comprising: said second primer including said second tag sequence (encompasses the step of appending second MID); and

sequences of said variable genetic region; mixing said first and second populations together; determining sequences of polynucleotides comprising tag sequences and the sequences of the variable genetic element in said mixture; from the tag sequences comprised in the polynucleotide sequences thus determined identifying the sample in which polynucleotide sequences occurred; from the sequences of the variable genetic region comprised in the polynucleotide sequences thus determined identifying particular variants of said variable genetic element; from this information determining the number of time one or more given variants occur in each sample, and from the number for each variant in the polynucleotides thus determined, quantifying said polynucleotides in said samples (Fig. 1 and its legend in pgs. 13 and 14 and Gillevet claim 1). Gillevet also teaches that the tags are comprised in primers for PCR amplification (pg. 18, lines 14 and 15), which meets the limitation of first and second MID sequences are appended during an amplification reaction to form the first and second appended segments.

Gillevet does not specifically teach the nucleic acid genome is from single isolated cells. However, nucleic acid genome isolated from single isolated cell was known in the art at the time the claimed invention was made as taught by Wangh, who teaches a method, wherein nucleic acid molecules are isolated from single cell from a sample (Wangh claims 25 and 45) and further teaches that the nucleic acid content from the single cell are useful for a variety of uses, including research, forensic science and diagnostic applications (paragraphs 0187-0202), thus providing motivation to include to use of isolated single cell from the sample(s) of Wangh in the method of Gillevet.

One having ordinary skill in the art would also recognize that while combining the step of Wangh in the method of Gillevet some routine optimization is necessary which is within the skills of one having ordinary skill in the art and motivation to include the missing step of use of isolated single cell comes from Wangh and not gleaned from the instant invention. One having ordinary skill in the art would be motivated to analyze the nucleic acid sequences from two different single cells rather than from two different samples because that would allow the monitor nucleic acid differences from individual genome of the cell.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the step of analyzing nucleic acid molecules from individual single cell of Wangh in the method of Gillevet with a reasonable expectation of success with the expected benefit of analyzing the nucleic acid sequences from individual isolated cells having a variety of uses, including research, forensic science and diagnostic applications as taught by Wangh. An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including the two single cell analysis which is routinely done in the art as exemplified by Wangh.

The teachings of Gillevet in view of Wangh regarding dependent claims 41-55 are discussed below.

Regarding claim 41, Gillevet teaches an amplification of plurality of nucleic acid attached to the MID sequence before determining the nucleic acid sequence (pg. 18, lines 14-18).

Regarding claims 42 and 43, Gillevet teaches that the nucleic acids are DNA (pg. 18, line 25) and RNA (pg. 9, lines 24 and 25). Wangh teaches nucleic acid molecule is mRNA (paragraph 0046).

Regarding claim 44, Gillevet teaches that the tagging step comprises ligation with nucleic acid (pg.8, lines 10-12).

Regarding claims 45 and 47, as discussed above while rejecting claim 40, Gillevet teaches that the tagging step comprises performing PCR of the plurality of nucleic acids and the MID sequence is in the primer (pg. 18, lines 12-18).

Regarding claim 46, as discussed above while rejecting claim 40, Gillevet in view of Wangh teaches providing a plurality of nucleic acids from each of a plurality of single cell sources; the attaching step comprises attaching different MID sequences to the nucleic acids from each of the plurality of single cell sources; and wherein nucleic acid sequences are attributed to different individual cell sources based upon the MID sequences attached in the attaching step and a sequencing primer sequence (Gillevet Fig. 1 and its legend in pgs. 13 and 14 and Gillevet claim 1 and Wangh claims 25 and 45).

Regarding claim 48, Gillevet teaches that the MID sequences (i.e., the tag sequences) are 2, 3, 4, 5, 6, 8, 10, 12 bases long, which is within the range of 2 to 100 bases in length (pg. 13, lines 16-18).

Regarding claims 49 and 50, Gillevet teaches that the nucleic acid segments are in the range of about 300 to 1700 bp (Fig. 4), which is in the claimed range of 100 to 5000 bp.

Regarding claim 51, Gillevet teaches a plurality of samples in the range of 5 to 10,000 samples, wherein each sample comprises a specific sequence tag (pg. 8, lines 3-9). One having ordinary skill in the art would recognize that the steps of claim 51 are obvious over Gillevet in view of Wangh because as discussed above while rejecting claim 40, Wangh provide motivation to include isolated single cell from each sample of Gillevet.

Regarding claim 52, Gillevet teaches samples are fragmented genomic DNAs (pg. 18, line 25) and further teaches amplified fragment is about 1700 bp (Fig. 4). Also the genomic fragment size in the range of 10-30 kb is routine, well established and conventional in the art as further evidenced by Drmanac (cited in the previous office action, paragraph 0023) and therefore obvious over Gillevet in view of Wangh.

Regarding claims 53-55, it is noted that claims 54 and 55 further specifies plurality of single isolated cells comprise first and second single isolated cells, wherein each cell type comprise specific MID sequence. As discussed above while rejecting claim 40, the subject matter of analyzing the first contiguous nucleic acid sequence from first single isolated cell and second contiguous nucleic acid sequence from second single isolated cell using MID sequence for each single isolated cell is obvious over Gillevet in view of Wangh for the reasons discussed above. Therefore the subject matter of claims 53-55 are obvious improvements over Gillevet in view of Wangh and therefore not novel.

Double Patenting

13. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the claims at issue are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); In re Vogel, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l) (1) - 706.02(l) (3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

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

14. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-12 of U.S. Patent No. 8,679,756 in view of Gillevet (WO 2008/061193, cited in the previous office action) and Wangh et al (US 2003/0022231 published Jan 30, 2003) for the following reasons.

Regarding instant independent claims 40 and 53, claims 1, 2 and 11 of '756 patent discloses a method comprising: a) obtaining a composition comprising a mixture

of: i. a first plurality of different segments of a first nucleic acid molecule of a contiguous region in genomic DNA, wherein said contiguous region in genomic DNA is represented by a plurality of nucleic acid molecules, and wherein each of said first plurality of segments is appended to a first multiplex identifier (MID) sequence; and ii. a second plurality of different segments of a second nucleic acid molecule of said contiguous region, wherein each of said second plurality of segments is appended to a second MID sequence and said first MID sequence and said second MID sequence are different, wherein, in said composition, all of the segments of each of the nucleic acid molecules of the contiguous region comprises a unique MID sequence that uniquely identifies each of the nucleic acid molecules; and b) sequencing said first plurality of segments and said a second plurality of segments to provide a plurality of sequences; wherein each sequence comprises: (i) the nucleotide sequence of at least a portion of a segment and (ii) an associated MID sequence, wherein said genomic DNA is genomic DNA from a virus or a bacterium, wherein defining a linkage relationship between said sequences using the MID sequence that is associated with each of the sequences, wherein sequences that comprise the first MID sequence are linked and sequences that comprise the second MID sequence are linked (common steps between instant claims 40 and 53 are underlined by the examiner). One having ordinary skill in the art would recognize that genomic DNA from a bacterium amounts of a genome from a single isolated cell.

However, claims of '756 patent do not disclose nucleic acid segments are from first and second isolated single cell. However, providing the genomic DNA from two

different cell types was known in the art at the time the claimed invention was made as taught by Gillevet, who teaches multiplex method for the quantitative determination of polynucleotides in two or more samples, wherein plurality of nucleic acids from first and second sample are appended with tag sequence during amplification, wherein the first tag sequence is different from the second tag sequence and determining the sequences of the polynucleotides including the tag sequences and identifying the variable genetic element in each sample based on tag sequence that identifies the sample type (Gillevet, claims 2 and 6), which are useful for detecting, monitoring, profiling, prognosticating, and/or diagnosing a disorder, disease, or the like (pg. 18, lines 31 and 32).

Claims of '756 patent in view of Gillevet does not specifically teach that the nucleic acids are from a genome from the single isolated cell, which is taught by Wangh, who teaches method, wherein nucleic acid molecules are isolated from single cell (Wangh claim 2) and further teaches that the nucleic acid content from the single cell are useful for a variety of uses, including research, forensic science and diagnostic applications (paragraphs 0187-0202).One having ordinary skill in the art would also recognize that while combining the steps of '756 patent with the step of Gillevet and Wangh some routine optimization is necessary which is within the skills of one having ordinary skill in the art and motivation to include the missing step comes from Gillevet and Wangh. One having ordinary skill in the art would be motivated to analyze the nucleic acid sequences from two different single cells rather than from plurality of different samples because that would allow the monitor nucleic acid differences from individual genome.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the step of analyzing nucleic acid molecules from a plurality of samples using tag sequence specific to each sample type of Gillevet and nucleic acid from individual single cell of Wangh in the method of '756 patent with a reasonable expectation of success with the expected benefit of analyzing the nucleic acid sequences from individual isolated cells having a variety of uses, including research, forensic science and diagnostic applications as taught by Gillevet and Wangh. An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including the two single cell analysis which is routinely done in the art and therefore the steps of instant claims 40 and 53 are not patentably distinct from claims of '756 patent in view of Gillevet and Wangh.

With regard to instant dependent claims 41-52, 54 and 55, claims of '756 patent in view of Gillevet and Wangh disclose the steps and therefore are not patentably distinct.

15. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-11 of U.S. Patent No. 8,563,274 in view of Gillevet (WO 2008/061193, cited in the previous office action) and Wangh et al (US 2003/0022231 published Jan 30, 2003) for the following reasons.

Regarding instant claims 40 and 53, claims 1 and 10 of '274 patent is drawn to a composition comprising a mixture of: a) a first plurality of different segments of a first nucleic acid molecule of a contiguous region in genomic DNA, wherein said contiguous

region in genomic DNA is represented by a plurality of nucleic acid molecules, and wherein each of said first plurality of segments is appended to a first multiplex identifier (MID) sequence; and b) a second plurality of different segments of a second nucleic acid molecule of said contiguous region, wherein each of said second plurality of segments is appended to a second MID sequence and said first MID sequence and said second MID sequence are different, wherein, in said composition, all of the segments of each of the nucleic acid molecules of the contiguous region comprise a unique MID sequence that uniquely identifies each of the nucleic acid molecules, wherein said genomic DNA is genomic DNA from a virus or a bacterium.

One having ordinary skill in the art would recognize that genomic DNA from a bacterium amounts of a genome from a single isolated cell. One having ordinary skill in the art would also recognize that providing a genome from a second isolated cell would be a duplication of the steps of '274 patent because instant claims as recited merely require analysis of the first and second appended segments and not linking the sequence to the first and the second isolated single cell.

However, claims of '274 patent do not disclose nucleic acid segments are from first and second isolated single cell. However, providing the genomic DNA from two different cell types was known in the art at the time the claimed invention was made as taught by Gillevet, who teaches multiplex method for the quantitative determination of polynucleotides in two or more samples, wherein plurality of nucleic acids from first and second sample are appended with tag sequence during amplification, wherein the first tag sequence is different from the second tag sequence and determining the sequences

of the polynucleotides including the tag sequences and identifying the variable genetic element in each sample based on tag sequence that identifies the sample type (Gillevet, claims 2 and 6), which are useful for detecting, monitoring, profiling, prognosticating, and/or diagnosing a disorder, disease, or the like (pg. 18, lines 31 and 32).

Claims of '274 patent in view of Gillevet does not specifically teach that the nucleic acids are from a genome from the single isolated cell, which is taught by Wangh, who teaches method, wherein nucleic acid molecules are isolated from single cell (Wangh claim 2) and further teaches the nucleic acid content from the single cell are useful for a variety of uses, including research, forensic science and diagnostic applications (paragraphs 0187-0202).One having ordinary skill in the art would also recognize that while combining the steps of '756 patent with the step of Gillevet and Wangh some routine optimization is necessary which is within the skills of one having ordinary skill in the art and motivation to include the missing step comes from Gillevet and Wangh. One having ordinary skill in the art would be motivated to analyze the nucleic acid sequences from two different single cells rather than from plurality of different samples because that would allow the monitor nucleic acid differences from individual genome.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the step of analyzing nucleic acid molecules from a plurality of samples using tag sequence specific to each sample type of Gillevet and nucleic acid from individual single cell of Wangh in the method of '274 patent with a reasonable expectation of success with the expected benefit of analyzing

the nucleic acid sequences from individual isolated cells having a variety of uses, including research, forensic science and diagnostic applications as taught by Gillevet and Wangh. An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including the two single cell analysis which is routinely done in the art and therefore the steps of instant claims 40 and 53 are not patentably distinct from claims of '274 patent in view of Gillevet and Wangh.

With regard to instant dependent claims 41-52, 54 and 55, claims of '274 patent in view of Gillevet and Wangh disclose the steps and therefore are not patentably distinct.

16. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-13 of U.S. Patent No. 9,410,149 in view of Gillevet (WO 2008/061193, cited in the previous office action) and Wangh et al (US 2003/0022231 published Jan 30, 2003) for the following reasons.

The subject matter of claims 1-13 of '149 patent is similar to claims 1-11 of '756 patent. Therefore it is maintained that steps of instant claims 40-55 are not significantly different from claims 1-13 of '149 patent in view of Gillevet and Wangh.

17. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-19 of U.S. Patent No. 9,410,150 in view of in view of Gillevet (WO 2008/061193, cited in the previous office action) and Wangh et al (US 2003/0022231 published Jan 30, 2003) for the following reasons.

The subject matter of claims 1-19 of '150 patent is similar to claims 1-11 of '274 patent. Therefore it is maintained that steps of instant claims 40-55 are not significantly different from claims 1-19 of '150 patent in view of Gillevet and Wangh.

Conclusion

18. No claims are allowed.

19. 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 NARAYAN BHAT whose telephone number is (571)272-5540. The examiner can normally be reached on 8.30 am to 7 pm.

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

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave Nguyen can be reached on (571)-272-0731. 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.

/NARAYAN BHAT/
Primary Examiner, Art Unit 1634

Notice of References Cited	Application/Control No. 14/792,094	Applicant(s)/Patent Under Reexamination BRENNER ET AL.	
	Examiner NARAYAN BHAT	Art Unit 1634	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-2003/0022231 A1	01-2003	Wangh, Lawrence	C12Q1/6806	435/6.11
	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	
	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.

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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	4	(brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "10X GENOMICS".as. or "COMPASS GENETICS".as. or "population genetics".as.) and isolat$5 near5 (single or individual or one) near5 cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 09:47
L2	20	(brenner.in. or mikawa.in. or osborne.in or slatter.in. or "10X GENOMICS".as. or "COMPASS GENETICS".as. or "population genetics".as.) and isolat$5 same (single or individual or one) same cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 09:59
L3	0	2 and genomic same (dna or nucleic or polynucleotide) same cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:02
L4	1	2 and genom$3 same (dna or nucleic or polynucleotide).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:03
L5	2389	genomic same (dna or nucleic or polynucleotide) same cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:04
L6	107	5 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier" "bar code" or tag or addressable) same (dna or nucleic or polynucleotide).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:06
L7	26	6 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier" "bar code" or tag or addressable) same (different or distinct or distiguish$4) same (origin or cell or sample or library).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:08
L8	298	("multiplex identifier" or barcode or	US-PGPUB;	OR	ON	2017/05/25

		"unique tag" or "unique identifier" "bar code" or tag or addressable) near5(different or distinct or distiguish$4) near5 (origin or cell or sample or library).clm.	USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			10:11
L9	103	8 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier" "bar code" or tag or addressable) near5 (dna or nucleic or polynucleotide).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:12
L10	7298	(dna or nucleic) near5 (single or individual or one) near5 cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:22
L11	36	10 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier" "bar code" or addressable) near5 (dna or nucleic or polynucleotide).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:23
L12	911	(isolat$3 or extract$3 or purif$5)near5 (dna or nucleic) near5 ("single cell" or "individual cell" or "one cell")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:58
L13	66	12 and(isolat$3 or extract$3 or purif$5)near5 (dna or nucleic) near5 ("single cell" or "individual cell" or "one cell").clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:58
S1	29794	brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "10X GENOMICS".as. or "COMPASS GENETICS".as. or "population genetics".as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 10:50
S2	134	S1 and (single or individual or one) near3 cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 10:51
S3	536	S1 and (dna or nucleic or polynucleotide) same (pcr or amplif$7)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT;	OR	ON	2017/01/23 10:54

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			IBM_TDB			
S4	18	S3 and (adapt$3 or linker) near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 10:56
S5	4	S4 and sequencing same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 10:58
S6	139392	(single or individual or one) near3 cell same (dna or nucleic or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:01
S7	3492	S6 and (adapt$3 or linker) near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:02
S8	872	S7 and sequencing same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:02
S9	88	S8 and (single or individual or one) near3 cell near5 first	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:03
S10	84	S8 and (single or individual or one) near3 cell near5 second	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:03
S11	66	S10 and first near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:05
S12	47	S11 and second near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO;	OR	ON	2017/01/23 11:05

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			JPO; DERWENT; IBM_TDB			
S13	43263	"first cell" and "second cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:15
S14	1761	S13 and (dna or nucleic or polynucleotide) same "first cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:16
S15	1549	S14 and (dna or nucleic or polynucleotide) same "second cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:17
S16	143	S15 and (adapt$3 or linker) same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:18
S17	27	S16 and second near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier) near5 first	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:19
S18	35	S16 and sequencing same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:20
S19	870	(single or individual or one) near3 cell near5 isolat$4 near5 first	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:29
S20	315	S19 and (single or individual or one) near3 cell near5 isolat$4 near5 second	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:30
S21	120	S20 and (dna or nucleic or polynucleotide) near5 cell	US-PGPUB; USPAT;	OR	ON	2017/01/23 11:31

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			USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			
S22	25	S21 and (adaptor or adapter or linker) same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:31
S23	12	S21 and sequencing same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:34
S24	540	sequencing same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence same cell near5 (type or individual$3 or single)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:36
S25	381	S24 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence same (identif$4 or detect$3 or dtermin$3) same cell near5 (type or individual$3 or single)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:37
S26	67	S25 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence near5 (identif$4 or detect$3 or dtermin$3) same cell adj (type or individual$3 or single)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:38
S27	2799	"first cell" same (nucleic or dna or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:47
S28	1549	S27 and "second cell" same (nucleic or dna or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:48
S29	1	S28 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence near5 (identif$4 or detect$3 or determin$3) near5 "first cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:49

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S30	8	S27 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence same (identif$4 or detect$3 or determin$3) near5 "first cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:51
S36	2321	(mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near5 sequence near5 (identif$4 or detect$3 or determin$3 or distinguish$3) near5 (cell or sample)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:59
S37	46	S36 and (append$3 or ligat$3) near5 (adpator or adapter or linker) near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier) same (dna or nucleic or polynucleotide) near5 (cell or sample)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 12:02
S38	21	(brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "10X GENOMICS".as. or "COMPASS GENETICS".as. or "population genetics".as.) and (nucleic or dna or polynucleotide)same (adaptor or adapter or linker) same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:32
S39	8	S38 and sequencing.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:32
S40	1	S39 and (single or individual or one) near3 cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:34
S41	134	(brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "10X GENOMICS".as. or "COMPASS GENETICS".as. or "population genetics".as.) and (single or individual or one) near3 cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:35
S42	3	S41 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)same sequence same (identif$4 or detect$3 or determin$3 or distinguish$3) same cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:36
S43	29	"9410150"	US-PGPUB; USPAT;	OR	ON	2017/01/23 18:04

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			USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			
S44	8	"9410149".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 18:05
S45	3	"8563274".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 18:06
S46	2	"8679756".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 18:08
S47	239	"multiplex identifier"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:33
S48	16	S47 and ("single cell" or "Individual cell") near5 (dna or nucleic)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:34
S49	12	"multiplex identifier" near5 (adaptor or linker or adapter)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:35
S50	56	"multiplex identifier" same (adaptor or linker or adapter)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:40
S51	5080	("multiplex identifier" or barcode or "unique tag" or "unique identifier")same (adaptor or linker or adapter)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:49

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S52	233	S51 and ("single cell" or "Individual cell") near5 (dna or nucleic)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:50
S53	226	S52 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier")same (dna or nucleic)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:50
S54	166	S53 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier")near5 sequence same(identif$4 or detect$3 or determin$3 or distinguish$3)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:52
S55	11	S54 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier")near5 contiguous	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:53
S56	56	S51 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier")near5 contiguous	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:53
S57	3	S51 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier")same contiguous near5 genomic near5 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:57
S58	13	("multiplex identifier" or barcode or "unique tag" or "unique identifier")same contiguous near5 genomic near5 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:58
S59	2	("multiplex identifier" or barcode or "unique tag" or "unique identifier")near5 contiguous near5 genomic	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:59
S60	179	("multiplex identifier" or barcode or "unique tag" or "unique identifier")near5 genomic near5 (dna or nucleic)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO;	OR	ON	2017/01/24 15:00

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			DERWENT; IBM_TDB			
S61	91	S60 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier")near5 sequence same(identif$4 or detect$3 or determin$3 or distinguish$3)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 15:00

5/ 25/ 2017 11:02:58 AM
C:\ Users\ nbhat1\ Documents\ EAST\ Workspaces\ 14792094-1-23-17.wsp

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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14792094	BRENNER ET AL.
	Examiner	Art Unit
	NARAYAN BHAT	1634

CPC- SEARCHED		
Symbol	Date	Examiner
C12Q 1/68, C12Q 1/6874, C07H 21/02 and C40B 30/04	1/6/16	Bhat N.
Searched uisng key words in different CPC classes. See the search history in EAST.	1/6/16	Bhat N.

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Reviewed the copending applications for the inventors in PLAM and copending claims for potential ODP issues.	1/6/16	Bhat N.
Reviewed the prosecution histories of copending applications resulting in the issuance of patents.	1/6/16	Bhat N.
Reviewed the cited arts in the IDS of 10/9/15.	1/6/16	Bhat N.
searched for the topics in EAST, Pubmed and Google scholar. Search histories are enclosed.	1/6/16	Bhat N.
Updated the search for the topics in EAST. Search history is enclosed.	7/17/16	Bhat N.
C12Q 1/68, C12Q 1/6874, C07H 21/02 and C40B 30/04	1/6/16	Bhat N.
Updated the search for the topics in EAST, Pubmed and Google scholar. Search histories are enclosed.		
Updated the search for the topics in EAST. Search history is enclosed.	5/25/17	Bhat N.

INTERFERENCE SEARCH

	/NARAYAN BHAT/ Primary Examiner.Art Unit 1634

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

	/NARAYAN BHAT/ Primary Examiner.Art Unit 1634

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Doc code: RCEX
Doc description: Request for Continued Examination (RCE)

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	14/792,094	Filing Date	2015-07-06	Docket Number (if applicable)	104371-5016-US05	Art Unit	1634
First Named Inventor	Sydney Brenner			Examiner Name	Narayan Kameshwar Bhat		

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 order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

- ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
- ☐ Other _____

☒ Enclosed

- ☒ Amendment/Reply
- ☒ Information Disclosure Statement (IDS)
- ☐ Affidavit(s)/ Declaration(s)
- ☐ Other _____

MISCELLANEOUS

☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____ (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

☐ Other _____

FEES

☒ **The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.**
The Director is hereby authorized to charge any underpayment of fees, or credit any overpayments, to Deposit Account No 500310

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

☒ Patent Practitioner Signature

Applicant Signature

EFS - Web 2.1.15

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

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Signature of Registered U.S. Patent Practitioner			
Signature	/Gargi Talukder/	Date (YYYY-MM-DD)	2017-08-30
Name	Gargi Talukder	Registration Number	61368

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450.

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

Privacy Act Statement

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

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

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

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

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

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

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

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

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

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

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

ftIN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 9697
Sidney Brenner et al.	Examiner: Narayan Kameshwar Bhat
Application No.: 14/792,094	Art Unit: 1634
Filed: July 6, 2015	
For: COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID ARRANGEMENT	RESPONSE TO FINAL OFFICE ACTION

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

Commissioner:

In response to the Final Office Action mailed May 31, 2017, please enter the following amendments and remarks:

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

Remarks/Arguments begin on page **4** of this paper.

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Amendments to the Claims:

This listing of claims will replace all prior versions, and listings of claims in the application:

Listing of Claims:

1-55. (Cancelled)

56. (New) A method of analyzing nucleic acids, the method comprising:

(a) providing a plurality of nucleic acid samples, each made from single isolated cells;

(b) tagging a plurality of segments of the nucleic acid molecules in the samples of (a) with first and second multiplex identifier (MID) sequences, wherein:

(i) the first MID sequences associated with a segment of a nucleic acid molecule of (a) uniquely identifies the single isolated cell from which the nucleic acid molecule is provided; and

(ii) the second MID sequences are all different from each other;

(c) sequencing the tagged segments of (b) to obtain the nucleotide sequences of the plurality of nucleic acids and the attached first and second MID sequences.

57. (New) The method of claim 56, wherein the nucleic acids are mRNA.

58. (New) The method of claim 56, wherein the method further comprises amplifying the segments and the appended MID sequences before the sequencing step (c).

59. (New) The method of claim 56, wherein the MID sequences are appended to the segments through ligation.

60. (New) The method of claim 56, wherein the MID sequences are appended to the segments by performing PCR or linear amplification of the plurality of nucleic acids using an adapter sequence comprising the MID sequence and an amplification primer.

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61. (New) The method of claim 56, wherein the MID sequences further comprise a sequencing primer sequence.

62. (New) The method of claim 56, wherein the MID sequences are from 2 to 100 bases in length.

63. (New) The method of claim 56, wherein the segments of (a) and (b) are in the range of 100 to 5,000 bp in length.

64. (New) The method of claim 40, wherein the segments are 400 bp or less in length.

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Remarks/Arguments

Applicants have not dedicated or abandoned any unclaimed subject matter and have not acquiesced to any rejections made by the Patent Office. Applicants reserve the right to pursue prosecution of any presently excluded claim embodiments in future continuation and/or divisional applications.

Status of the Claims

Claims 56-64 are newly added and are fully supported by the specification and claims as originally filed.

Applicants respectfully request entry of the claims as amended into the record.

Response to Office Action

Claim Rejections – 35 USC § 103

Claims 40-55 are rejected under 35 U.S.C. § 103 as being allegedly anticipated by Gillevet, WO 2008/061193 ("Gillevet") in view of Wangh et al., US 2003/0022231 ("Wangh"). Applicants respectfully traverse these rejections.

Applicants submit that the combined teachings of Gillevet and Wangh fail to teach all elements of the claimed invention. In particular, the combined teachings of these references fail to teach methods of analyzing nucleic acids from single isolated cells, where segments of the nucleic acids are appended to two MID sequences and where the first MID sequence identifies the source of the nucleic acid segments and the second MID sequence is unique from the other second MID sequences and can be used for further deep analysis of the sample sequences, including quantification. Neither Gillevet or Wangh alone or in combination teaches this element of the claimed invention.

Applicants further note that Gillevet teaches a method for sample indexing – such a method would have no need for a second MID sequence as recited in the present claims. In

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addition, Wangh is unrelated to a sequencing method – the methods taught in Wangh are real-time PCR assay methods that answer a "yes or no" question, i.e., whether a particular sequence is present in a sample. There is no analysis of segments appended to two MID sequences as recited in the present claims. As such, the skilled artisan would have no reasonable expectation of success in practicing the claimed invention based on the combined teachings of Gillevet and Wangh, because not only do both references lack elements of the claimed invention, but also the teachings in the two references are incompatible with each other.

For at least the foregoing reasons, Applicants respectfully submit that an obviousness rejection cannot be maintained based on the teachings of Gillevet and Wangh and request that all rejections under § 103 be withdrawn.

Claim Rejections – Double Patenting

Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-12 of U.S. Patent No. 8,679,756 in view of Gillevet, WO 2008/061193 ("Gillevet") and Wangh et al., US 2003/0022231. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-11 of U.S. Patent No. 8,563,274 in view of Gillevet, WO 2008/061193 ("Gillevet") and Wangh et al., US 2003/0022231. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-19 of U.S. Patent No. 9,410,150 in view of Gillevet, WO 2008/061193 ("Gillevet") and Wangh et al., US 2003/0022231.

For the reasons recited above, Applicants respectfully submit the presently claimed invention does not support a rejection on the ground of nonstatutory double patenting over any of the cited patents, as the present claims are not obvious in light of U.S. Patent No. 8,679,756 in view of Gillevet and Wangh. Applicants respectfully request that all double patenting rejections be withdrawn.

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CONCLUSION

All the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn.

Applicants believe that a full and complete reply has been made to the outstanding Office Action, and as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided below.

Except for issue fees payable under 37 C.F.R. § 1.18, the Commissioner is hereby authorized by this paper to charge any necessary fees during the entire pendency of this application including fees due under 37 C.F.R. §§ 1.16 and 1.17, which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-0310 (Order No.: 104371-5016-US05).

Prompt and favorable consideration of this Amendment and Response is respectfully requested.

Respectfully submitted,
MORGAN, LEWIS & BOCKIUS LLP

Dated: August 30, 2017 By: /Gargi Talukder/
Gargi Talukder, Reg. No. 61,368

Customer No.: 43850
Morgan, Lewis & Bockius LLP
One Market, Spear Street Tower
San Francisco, CA 94105
Telephone: (415) 442-1000
Facsimile: (415) 442-1001

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(71) Applicants *(for all designated States except US)*: **LIVING MICROSYSTEMS, INC.** [US/US]; 480 Arsenal Street, Suite 130, Watertown, MA 02472 (US). **CELLPOINT DIAGNOSTICS, INC.** [US/US]; 505 Coast Blvd. South, Suite 406, La Jolla, CA 92037 (US). **THE GENERAL HOSPITAL CORPORATION** [US/US]; 55 Fruit Street, Boston, MA 02114 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SHOEMAKER, Daniel** [US/US]; 5481 Caminito Exquisito, San Diego, CA 92130 (US). **FUCHS, Martin** [DK/US]; 11 Sophia Drive, Uxbridge, MA 01569 (US). **KRUEGER, Neil, X.** [US/US]; 776 Centre Street, Jamaica Plain, MA 02130 (US). **TONER, Mehmet** [US/US]; 100 Pilgrim Road, Wellesley Hills, MA 02481 (US). **GRAY, Darren** [US/US]; 211 Saint Paul Street, #2, Brookline, MA 02446 (US). **KAPUR, Ravi** [US/US]; 292 Morton Street, Stoughton, MA 02072 (US). **WANG, Zihua** [US/US]; 115 Synthia Road, Newton, MA 02459 (US).

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

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(54) Title: RARE CELL ANALYSIS USING SAMPLE SPLITTING AND DNA TAGS

(57) Abstract: The present invention provides systems, apparatuses, and methods to detect the presence of fetal cells when mixed with a population of maternal cells in a sample and to test fetal abnormalities, e.g. aneuploidy. The present invention involves labeling regions of genomic DNA in each cell in said mixed sample with different labels wherein each label is specific to each cell and quantifying the labeled regions of genomic DNA from each cell in the mixed sample. More particularly the invention involves quantifying labeled DNA polymorphisms from each cell in the mixed sample.

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RARE CELL ANALYSIS USING SAMPLE SPLITTING AND DNA TAGS

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 60/804,819, filed June 14, 2006, which application is incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] Analysis of specific cells can give insight into a variety of diseases. These analyses can provide non-invasive tests for detection, diagnosis and prognosis of diseases such as cancer or fetal disorders, thereby eliminating the risk of invasive diagnosis. Regarding fetal disorders, current prenatal diagnosis, such as amniocentesis and chorionic villus sampling (CVS), are potentially harmful to the mother and to the fetus. The rate of miscarriage for pregnant women undergoing amniocentesis is increased by 0.5-1%, and that figure is slightly higher for CVS. Because of the inherent risks posed by amniocentesis and CVS, these procedures are offered primarily to older women, e.g., those over 35 years of age, who have a statistically greater probability of bearing children with congenital defects. As a result, a pregnant woman at the age of 35 has to balance an average risk of 0.5-1% to induce an abortion by amniocentesis against an age related probability for trisomy 21 of less than 0.3%.

[0003] Regarding prenatal diagnostics, some non-invasive methods have already been developed to screen for fetuses at higher risk of having specific congenital defects. For example, maternal serum alpha-fetoprotein, and levels of unconjugated estriol and human chorionic gonadotropin can be used to identify a proportion of fetuses with Down's syndrome. However, these tests suffer from many false positive. Similarly, ultrasonography is used to determine congenital defects involving neural tube defects and limb abnormalities, but such methods are limited to time periods after fifteen weeks of gestation and are present unreliable results.

[0004] The presence of fetal cells within the blood of pregnant women offers the opportunity to develop a prenatal diagnostic that replaces amniocentesis and thereby eliminates the risk of today's invasive diagnosis. However, fetal cells represent a small number of cells against the background of a large number of maternal cells in the blood which make the analysis time consuming and prone to error.

[0005] With respect to cancer diagnosis, early detection is of paramount importance. Cancer is a disease marked by the uncontrolled proliferation of abnormal cells. In normal tissue, cells divide and organize within the tissue in response to signals from surrounding cells. Cancer cells do not respond in the same way to these signals, causing them to proliferate and, in many organs, form a tumor. As the growth of a tumor continues, genetic alterations may accumulate, manifesting as a more aggressive growth phenotype of the cancer cells. If left untreated, metastasis, the spread of cancer cells to distant areas of the body by way of the lymph system or bloodstream, may ensue. Metastasis results in the formation of secondary tumors at multiple sites, damaging healthy tissue. Most cancer death is caused by such secondary tumors. Despite decades of advances in cancer diagnosis and therapy, many cancers continue to go undetected until late in their development. As one example, most early-stage lung cancers are asymptomatic and are not detected in time for curative treatment, resulting in an overall five-year survival rate for patients with lung cancer of less than 15%. However, in those instances in which lung cancer is detected and treated at an early stage, the prognosis is much more favorable.

[0006] The methods of the present invention allow for the detection of fetal cells and fetal abnormalities when fetal cells are mixed with a population of maternal cells, even when the maternal cells dominate the mixture. In addition, the methods of the present invention can also be utilized to detect or diagnose cancer.

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SUMMARY OF THE INVENTION

[0007] The present invention relates to methods for the detection of fetal cells or cancer cells in a mixed sample. In one embodiment, the present invention provides methods for determining fetal abnormalities in a sample comprising fetal cells that are mixed with a population of maternal cells. In some embodiments, determining the presence of fetal cells and fetal abnormalities comprises labeling one or more regions of genomic DNA in each cell from a mixed sample comprising at least one fetal cell with different labels wherein each label is specific to each cell. In some embodiments, the genomic DNA to be labeled comprises one or more polymorphisms, particularly STRs or SNPs

[0008] In some embodiments, the methods of the invention allow for simultaneously detecting the presence of fetal cells and fetal abnormalities when fetal cells are mixed with a population of maternal cells, even when the maternal cells dominate the mixture. In some embodiments, the sample is enriched to contain at least one fetal and one non fetal cell, and in other embodiments, the cells of the enriched population can be divided between two or more discrete locations that can be used as addressable locations. Examples of addressable locations include wells, bins, sieves, pores, geometric sites, slides, matrixes, membranes, electric traps, gaps, obstacles or in-situ within a cell or nuclear membrane.

[0009] In some embodiments, the methods comprise labeling one or more regions of genomic DNA in each cell in the enriched sample with different labels, wherein each label is specific to each cell, and quantifying the labeled DNA regions. The labeling methods can comprise adding a unique tag sequence for each cell in the mixed sample. In some embodiments, the unique tag sequence identifies the presence or absence of a DNA polymorphism in each cell from the mixed sample. Labels are added to the cells/DNA using an amplification reaction, which can be performed by PCR methods. For example, amplification can be achieved by multiplex PCR. In some embodiments, a further PCR amplification is performed using nested primers for the genomic DNA region(s).

[0010] In some embodiments, the DNA regions can be amplified prior to being quantified. The labeled DNA can be quantified using sequencing methods, which, in some embodiments, can precede amplifying the DNA regions. The amplified DNA region(s) can be analyzed by sequencing methods. For example, ultra deep sequencing can be used to provide an accurate and quantitative measurement of the allele abundances for each STR or SNP. In other embodiments, quantitative genotyping can be used to declare the presence of fetal cells and to determine the copy numbers of the fetal chromosomes. Preferably, quantitative genotyping is performed using molecular inversion probes.

[0011] The invention also relates to methods of identifying cells from a mixed sample with non-maternal genomic DNA and identifying said cells with non-maternal genomic DNA as fetal cells. In some embodiments, the ratio of maternal to paternal alleles is compared on the identified fetal cells in the mixed sample.

[0012] .In one embodiment, the invention provides for a method for determining a fetal abnormality in a maternal sample that comprises at least one fetal and one non fetal cell. The sample can be enriched to contain at least one fetal cell, and the enriched maternal sample can be arrayed into a plurality of discrete sites. In some embodiments, each discrete site comprises no more than one cell.

[0013] In some embodiments, the invention comprises labeling one or more regions of genomic DNA from the arrayed samples using primers that are specific to each DNA region or location, amplifying the DNA region(s), and quantifying the labeled DNA region. The labeling of the DNA region(s) can comprise labeling each region with a unique tag sequence, which can be used to identify the presence or absence of a DNA polymorphism on arrayed cells and the distinct location of the cells.

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[0014] The step of determining can comprise identifying non-maternal alleles at the distinct locations, which can result from comparing the ratio of maternal to paternal alleles at the location. In some embodiments, the method of identifying a fetal abnormality in an arrayed sample can further comprise amplifying the genomic DNA regions. The genomic DNA regions can comprise one or more polymorphisms e.g. STRs and SNPs, which can be amplified using PCR methods including multiplex PCR. An additional amplification step can be performed using nested primers.

[0015] The amplified DNA region(s) can be analyzed by sequencing methods. For example, ultra deep sequencing can be used to provide an accurate and quantitative measurement of the allele abundances for each STR or SNP. In other embodiments, quantitative genotyping can be sued to declare the presence of fetal cells and to determine the copy numbers of the fetal chromosomes. Preferably, quantitative genotyping is performed using molecular inversion probes.

[0016] In one embodiment, the invention provides methods for diagnosing a cancer and giving a prognosis by obtaining and enriching a blood sample from a patient for epithelial cells, splitting the enriched sample into discrete locations, and performing one or more molecular and/or morphological analyses on the enriched and split sample. The molecular analyses can include detecting the level of expression or a mutation of gene disclosed in Figure 10. Preferably, the method comprises performing molecular analyses on EGFR, EpCAM, GA733-2, MUC-1, HER-2, or Claudin-7 in each arrayed cell. The morphological analyses can include identifying, quantifying and /or characterizing mitochondrial DNA, telomerase, or nuclear matrix proteins.

[0017] In some embodiments, the sample can be enriched for epithelial cells by at least 10,000 fold, and the diagnosis and prognosis can be provided prior to treating the patient for the cancer. Preferably, the blood samples are obtained from a patient at regular intervals such as daily, or every 2, 3 or 4 days, weekly, bimonthly, monthly, bi-yearly or yearly.

[0018] In some embodiments, the step of enriching a patient's blood sample for epithelial cells involves flowing the sample through a first array of obstacles that selectively directs cells that are larger than a predetermined size to a first outlet and cells that are smaller than a predetermined size to a second outlet. Optionally, the sample can be subjected to further enrichment by flowing the sample through a second array of obstacles, which can be coated with antibodies that selectively bind to white blood cells or epithelial cells. For example, the obstacles of the second array can be coated with anti-EpCAM antibodies.

[0019] Splitting the sample of cells of the enriched population can comprises splitting the enriched sample to locate individual cells at discrete sites that can be addressable sites. Examples of addressable locations include wells, bins, sieves, pores, geometric sites, slides, matrixes, membranes, electric traps, gaps, obstacles or in-situ within a cell or nuclear membrane.

[0020] In some embodiments there are provided kits comprising devices for enriching the sample and the devices and reagents needed to perform the genetic analysis. The kits may contain the arrays for size-based separation, reagents for uniquely labeling the cells, devices for splitting the cells into individual addressable locations and reagents for the genetic analysis.

SUMMARY OF THE DRAWINGS

[0021] **Figures 1A-1D** illustrate various embodiments of a size-based separation module.

[0022] **Figures 2A-2C** illustrate one embodiment of an affinity separation module.

[0023] **Figure 3** illustrate one embodiment of a magnetic separation module.

[0024] **Figure 4** illustrates an overview for diagnosing, prognosing, or monitoring a prenatal condition in a fetus.

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[0025] **Figure 5** illustrates an overview for diagnosing, prognosing, or monitoring a prenatal condition in a fetus.

[0026] **Figure 6** illustrates an overview for diagnosing, prognosing or monitoring cancer in a patient.

[0027] **Figures 7A-7B** illustrate an assay using molecular inversion probes. Figure 7 C illustrates an overview of the use of nucleic acid tags.

[0028] **Figures 8A-8C** illustrate one example of a sample splitting apparatus.

[0029] **Figure 9** illustrates the probability of having 2 or more CTC's loaded into a single sample well.

[0030] **Figure 10** illustrates genes whose expression or mutations can be associated with cancer or another condition diagnosed herein.

[0031] **Figure 11** illustrates primers useful in the methods herein.

[0032] **Figure 12A-B** illustrate cell smears of the product and waste fractions.

[0033] **Figure 13A-F** illustrate isolated fetal cells confirmed by the reliable presence of male cells.

[0034] **Figure 14** illustrates cells with abnormal trisomy 21 pathology.

[0035] **Figure 15** illustrates performance of a size-based separation module.

[0036] **Figure 16** illustrates histograms of these cell fractions resulting from a size-based separation module.

[0037] **Figure 17** illustrates a first output and a second output of a size-based separation module.

[0038] **Figure 18** illustrates epithelial cells bound to a capture module of an array of obstacles coated with anti-EpCAM.

[0039] **Figures 19A-C** illustrate one embodiment of a flow-through size-based separation module adapted to separate epithelial cells from blood and alternative parameters that can be used with such device.

[0040] **Figure 20A-D** illustrate various targeted subpopulations of cells that can be isolated using size-based separation and various cut-off sizes that can be used to separate such targeted subpopulations.

[0041] **Figure 21** illustrates a device of the invention with counting means to determine the number of cells in the enriched sample.

[0042] **Figure 22** illustrates an overview of one aspect of the invention for diagnosing, prognosing, or monitoring cancer in a patient.

[0043] **Figure 23** illustrates the use of EGFR mRNA for generating sequencing templates.

[0044] **Figure 24** illustrates performing real-time quantitative allele-specific PCR reactions to confirm the sequence of mutations in EGFR mRNA.

[0045] **Figure 25** illustrates confirmation of the presence of a mutation is when the signal from a mutant allele probe rises above the background level of fluorescence.

[0046] **Figure 26A-B** illustrate the presence of EGFR mRNA in epithelial cells but not leukocytes.

[0047] **Figure 27** illustrate results of the first and second EGFR PCR reactions.

[0048] **Figure 28A-B** results of the first and second EGFR PCR reactions.

[0049] **Figure 29** illustrates that EGFR wild type and mutant amplified fragments are readily detected, despite the high leukocyte background.

[0050] **Figure 30** illustrates the detection of single copies of a fetal cell genome by qPCR.

[0051] **Figure 31** illustrates detection of single fetal cells in binned samples by SNP analysis.

[0052] **Figure 32** illustrates a method of trisomy testing. The trisomy 21 screen is based on scoring of target cells obtained from maternal blood. Blood is processed using a cell separation module for hemoglobin enrichment (CSM-HE). Enriched cells are transferred to slides that are first stained and subsequently probed by FISH. Images are acquired, such as from bright field or fluorescent microscopy, and scored. The proportion of trisomic cells of certain classes serves as a classifier for risk of fetal trisomy 21. Fetal genome identification can performed using

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assays such as: (1) STR markers; (2) qPCR using primers and probes directed to loci, such as the multi-repeat DYZ locus on the Y-chromosome; (3) SNP detection; and (4) CGH (comparative genome hybridization) array detection.

[0053] **Figure 33** illustrates assays that can produce information on the presence of aneuploidy and other genetic disorders in target cells. Information on aneuploidy and other genetic disorders in target cells may be acquired using technologies such as: (1) a CGH array established for chromosome counting, which can be used for aneuploidy determination and/or detection of intra-chromosomal deletions; (2) SNP/taqman assays, which can be used for detection of single nucleotide polymorphisms; and (3) ultra-deep sequencing, which can be used to produce partial or complete genome sequences for analysis.

[0054] **Figure 34** illustrates methods of fetal diagnostic assays. Fetal cells are isolated by CSM-HE enrichment of target cells from blood. The designation of the fetal cells may be confirmed using techniques comprising FISH staining (using slides or membranes and optionally an automated detector), FACS, and/or binning. Binning may comprise distribution of enriched cells across wells in a plate (such as a 96 or 384 well plate), microencapsulation of cells in droplets that are separated in an emulsion, or by introduction of cells into microarrays of nanofluidic bins. Fetal cells are then identified using methods that may comprise the use of biomarkers (such as fetal (gamma) hemoglobin), allele-specific SNP panels that could detect fetal genome DNA, detection of differentially expressed maternal and fetal transcripts (such as Affymetrix chips), or primers and probes directed to fetal specific loci (such as the multi-repeat DYZ locus on the Y-chromosome). Binning sites that contain fetal cells are then be analyzed for aneuploidy and/or other genetic defects using a technique such as CGH array detection, ultra deep sequencing (such as Solexa, 454, or mass spectrometry), STR analysis, or SNP detection.

Figure 35 illustrates methods of fetal diagnostic assays, further comprising the step of whole genome amplification prior to analysis of aneuploidy and/or other genetic defects.

INCORPORATION BY REFERENCE

[0055] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

DETAILED DESCRIPTION OF THE INVENTION

[0056] The present invention provides systems, apparatus, and methods to detect the presence of or abnormalities of rare analytes or cells, such as hematapoeitic bone marrow progenitor cells, endothelial cells, fetal cells, epithelial cells, or circulating tumor cells in a sample of a mixed analyte or cell population (e.g., maternal peripheral blood samples).

I. Sample Collection/Preparation

[0057] Samples containing rare cells can be obtained from any animal in need of a diagnosis or prognosis or from an animal pregnant with a fetus in need of a diagnosis or prognosis. In one example, a sample can be obtained from animal suspected of being pregnant, pregnant, or that has been pregnant to detect the presence of a fetus or fetal abnormality. In another example, a sample is obtained from an animal suspected of having, having, or an animal that had a disease or condition (e.g. cancer). Such condition can be diagnosed, prognosed, monitored and therapy can be determined based on the methods and systems herein. Animal of the present invention can be a human or a domesticated animals such as a cow, chicken, pig, horse, rabbit, dogs, cat, or goat. Samples derived from an animal or human can include, e.g., whole blood, sweat, tears, ear flow, sputum, lymph, bone marrow suspension, lymph, urine, saliva, semen, vaginal flow, cerebrospinal fluid, brain fluid, ascites, milk, secretions of the respiratory, intestinal or genitourinary tracts fluid.

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[0058] To obtain a blood sample, any technique known in the art may be used, e.g. a syringe or other vacuum suction device. A blood sample can be optionally pre-treated or processed prior to enrichment. Examples of pre-treatment steps include the addition of a reagent such as a stabilizer, a preservative, a fixant, a lysing reagent, a diluent, an anti-apoptotic reagent, an anti-coagulation reagent, an anti-thrombotic reagent, magnetic property regulating reagent, a buffering reagent, an osmolality regulating reagent, a pH regulating reagent, and/or a cross-linking reagent.

[0059] When a blood sample is obtained, a preservative such an anti-coagulation agent and/or a stabilizer is often added to the sample prior to enrichment. This allows for extended time for analysis/detection. Thus, a sample, such as a blood sample, can be enriched and/or analyzed under any of the methods and systems herein within 1 week, 6 days, 5 days, 4 days, 3 days, 2 days, 1 day, 12 hrs, 6 hrs, 3 hrs, 2 hrs, or 1 hr from the time the sample is obtained.

[0060] In some embodiments, a blood sample can be combined with an agent that selectively lyses one or more cells or components in a blood sample. For example, fetal cells can be selectively lysed releasing their nuclei when a blood sample including fetal cells is combined with deionized water. Such selective lysis allows for the subsequent enrichment of fetal nuclei using, e.g., size or affinity based separation. In another example platelets and/or enucleated red blood cells are selectively lysed to generate a sample enriched in nucleated cells, such as fetal nucleated red blood cells (fnRBC's), maternal nucleated blood cells (mnBC), epithelial cells and circulating tumor cells. fnRBC's can be subsequently separated from mnBC's using, e.g., antigen-i affinity or differences in hemoglobin

[0061] When obtaining a sample from an animal (e.g., blood sample), the amount can vary depending upon animal size, its gestation period, and the condition being screened. In some embodiments, up to 50, 40, 30, 20, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 mL of a sample is obtained. In some embodiments, 1-50, 2-40, 3-30, or 4-20 mL of sample is obtained. In some embodiments, more than 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 mL of a sample is obtained.

[0062] To detect fetal abnormality, a blood sample can be obtained from a pregnant animal or human within 36, 24, 22, 20, 18, 16, 14, 12, 10, 8, 6 or 4 weeks of gestation.

II. Enrichment

[0063] A sample (e.g. blood sample) can be enriched for rare analytes or rare cells (e.g. fetal cells, epithelial cells or circulating tumor cells) using one or more any methods known in the art (e.g. Guetta, EM et al. Stem Cells Dev, 13(1):93-9 (2004)) or described herein. The enrichment increases the concentration of rare cells or ratio of rare cells to non-rare cells in the sample. For example, enrichment can increase concentration of an analyte of interest such as a fetal cell or epithelial cell or CTC by a factor of at least 2, 4, 6, 8, 10, 20, 50, 100, 200, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 2,000,000, 5,000,000, 10,000,000, 20,000,000, 50,000,000, 100,000,000, 200,000,000, 500,000,000, 1,000,000,000, 2,000,000,000, or 5,000,000,000 fold over its concentration in the original sample. In particular, when enriching fetal cells from a maternal peripheral venous blood sample, the initial concentration of the fetal cells may be about 1:50,000,000 and it may be increased to at least 1:5,000 or 1:500. Enrichment can also increase concentration of rare cells in volume of rare cells / total volume of sample (removal of fluid). A fluid sample (e.g., a blood sample) of greater than 10, 15, 20, 50, or 100 mL total volume comprising rare components of interest, and it can be concentrated such that the rare component of interest into a concentrated solution of less than 0.5, 1, 2, 3, 5, or 10 mL total volume.

[0064] Enrichment can occur using one or more types of separation modules. Several different modules are described herein, all of which can be fluidly coupled with one another in the series for enhanced performance.

[0065] In some embodiments, enrichment occurs by selective lysis as described above.

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[0066] In one embodiment, enrichment of rare cells occurs using one or more size-based separation modules. Examples of size-based separation modules include filtration modules, sieves, matrixes, etc. Examples of size-based separation modules contemplated by the present invention include those disclosed in International Publication No. WO 2004/113877. Other size based separation modules are disclosed in International Publication No. WO 2004/0144651.

[0067] In some embodiments, a size-based separation module comprises one or more arrays of obstacles forming a network of gaps. The obstacles are configured to direct particles as they flow through the array/network of gaps into different directions or outlets based on the particle's hydrodynamic size. For example, as a blood sample flows through an array of obstacles, nucleated cells or cells having a hydrodynamic size larger than a predeterminedcertain size such as a cuttoff or predetermined size, e.g., 8 microns, are directed to a first outlet located on the opposite side of the array of obstacles from the fluid flow inlet, while the enucleated cells or cells having a hydrodynamic size smaller than a predetermined size, e.g., 8 microns, are directed to a second outlet also located on the opposite side of the array of obstacles from the fluid flow inlet.

[0068] An array can be configured to separate cells smaller or larger than a predetermined size by adjusting the size of the gaps, obstacles, and offset in the period between each successive row of obstacles. For example, in some embodiments, obstacles or gaps between obstacles can be up to 10, 20, 50, 70, 100, 120, 150, 170, or 200 microns in length or about 2, 4, 6, 8 or 10 microns in length. In some embodiments, an array for size-based separation includes more than 100, 500, 1,000, 5,000, 10,000, 50,000 or 100,000 obstacles that are arranged into more than 10, 20, 50, 100, 200, 500, or 1000 rows. Preferably, obstacles in a first row of obstacles are offset from a previous (upstream) row of obstacles by up to 50% the period of the previous row of obstacles. In some embodiments, obstacles in a first row of obstacles are offset from a previous row of obstacles by up to 45, 40, 35, 30, 25, 20, 15 or 10% the period of the previous row of obstacles. Furthermore, the distance between a first row of obstacles and a second row of obstacles can be up to 10, 20, 50, 70, 100, 120, 150, 170 or 200 microns. A particular offset can be continuous (repeating for multiple rows) or non-continuous. In some embodiments, a separation module includes multiple discrete arrays of obstacles fluidly coupled such that they are in series with one another. Each array of obstacles has a continuous offset. But each subsequent (downstream) array of obstacles has an offset that is different from the previous (upstream) offset. Preferably, each subsequent array of obstacles has a smaller offset that the previous array of obstacles. This allows for a refinement in the separation process as cells migrate through the array of obstacles. Thus, a plurality of arrays can be fluidly coupled in series or in parallel, (e.g., more than 2, 4, 6, 8, 10, 20, 30, 40, 50). Fluidly coupling separation modules (e.g., arrays) in parallel allows for high-throughput analysis of the sample, such that at least 1, 2, 5, 10, 20, 50, 100, 200, or 500 mL per hour flows through the enrichment modules or at least 1, 5, 10, or 50 million cells per hour are sorted or flow through the device.

[0069] **Figure 1A** illustrates an example of a size-based separation module. Obstacles (which may be of any shape) are coupled to a flat substrate to form an array of gaps. A transparent cover or lid may be used to cover the array. The obstacles form a two-dimensional array with each successive row shifted horizontally with respect to the previous row of obstacles, where the array of obstacles directs component having a hydrodynamic size smaller than a predetermined size in a first direction and component having a hydrodynamic size larger that a predetermined size in a second direction. For enriching epithelial or circulating tumor cells from enucleated, the predetermined size of an array of obstacles can be get at 6-12 μm or 6-8 μm. For enriching fetal cells from a mixed sample (e.g. maternal blood sample) the predetermined size of an array of obstacles can be between 4-10 μm or 6-8 μm. The flow of sample into the array of obstacles can be aligned at a small angle (flow angle) with respect to a line-of-sight of the array. Optionally, the array is coupled to an infusion pump to perfuse the sample through the obstacles. The flow

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conditions of the size-based separation module described herein are such that cells are sorted by the array with minimal damage. This allows for downstream analysis of intact cells and intact nuclei to be more efficient and reliable.

[0070] In some embodiments, a size-based separation module comprises an array of obstacles configured to direct cells larger than a predetermined size to migrate along a line-of-sight within the array (e.g. towards a first outlet or bypass channel leading to a first outlet), while directing cells and analytes smaller than a predetermined size to migrate through the array of obstacles in a different direction than the larger cells (e.g. towards a second outlet). Such embodiments are illustrated in part in **Figures 1B-1D**.

[0071] A variety of enrichment protocols may be utilized although gentle handling of the cells is needed to reduce any mechanical damage to the cells or their DNA. This gentle handling also preserves the small number of fetal or rare cells in the sample. Integrity of the nucleic acid being evaluated is an important feature to permit the distinction between the genomic material from the fetal or rare cells and other cells in the sample. In particular, the enrichment and separation of the fetal or rare cells using the arrays of obstacles produces gentle treatment which minimizes cellular damage and maximizes nucleic acid integrity permitting exceptional levels of separation and the ability to subsequently utilize various formats to very accurately analyze the genome of the cells which are present in the sample in extremely low numbers.

[0072] In some embodiments, enrichment of rare cells (e.g. fetal cells, epithelial cells or circulating tumor cells (CTCs)) occurs using one or more capture modules that selectively inhibit the mobility of one or more cells of interest. Preferable a capture module is fluidly coupled downstream to a size-based separation module. Capture modules can include a substrate having multiple obstacles that restrict the movement of cells or analytes greater than a predetermined size. Examples of capture modules that inhibit the migration of cells based on size are disclosed in U.S. Patent No. 5,837,115 and 6,692,952.

[0073] In some embodiments, a capture module includes a two dimensional array of obstacles that selectively filters or captures cells or analytes having a hydrodynamic size greater than a particular gap size (predetermined size), International Publication No. WO 2004/113877.

[0074] In some cases a capture module captures analytes (e.g., cells of interest or not of interest) based on their affinity. For example, an affinity-based separation module that can capture cells or analytes can include an array of obstacles adapted for permitting sample flow through, but for the fact that the obstacles are covered with binding moieties that selectively bind one or more analytes (e.g., cell populations) of interest (e.g., red blood cells, fetal cells, epithelial cells or nucleated cells) or analytes not-of-interest (e.g., white blood cells). Arrays of obstacles adapted for separation by capture can include obstacles having one or more shapes and can be arranged in a uniform or non-uniform order. In some embodiments, a two-dimensional array of obstacles is staggered such that each subsequent row of obstacles is offset from the previous row of obstacles to increase the number of interactions between the analytes being sorted (separated) and the obstacles.

[0075] Binding moieties coupled to the obstacles can include e.g., proteins (e.g., ligands/receptors), nucleic acids having complementary counterparts in retained analytes, antibodies, etc. In some embodiments, an affinity-based separation module comprises a two-dimensional array of obstacles covered with one or more antibodies selected from the group consisting of: anti-CD71, anti-CD235a, anti-CD36, anti-carbohydrates, anti-selectin, anti-CD45, anti-GPA, anti-antigen-i, anti-EpCAM, anti-E-cadherin, and anti-Muc-1.

[0076] **Figure 2A** illustrates a path of a first analyte through an array of posts wherein an analyte that does not specifically bind to a post continues to migrate through the array, while an analyte that does bind a post is captured by the array. **Figure 2B** is a picture of antibody coated posts. **Figure 2C** illustrates coupling of antibodies to a

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substrate (e.g., obstacles, side walls, etc.) as contemplated by the present invention. Examples of such affinity-based separation modules are described in International Publication No. WO 2004/029221.

[0077] In some embodiments, a capture module utilizes a magnetic field to separate and/or enrich one or more analytes (cells) based on a magnetic property or magnetic potential in such analyte of interest or an analyte not of interest. For example, red blood cells which are slightly diamagnetic (repelled by magnetic field) in physiological conditions can be made paramagnetic (attributed by magnetic field) by deoxygenation of the hemoglobin into methemoglobin. This magnetic property can be achieved through physical or chemical treatment of the red blood cells. Thus, a sample containing one or more red blood cells and one or more white blood cells can be enriched for the red blood cells by first inducing a magnetic property in the red blood cells and then separating the red blood cells from the white blood cells by flowing the sample through a magnetic field (uniform or non-uniform).

[0078] For example, a maternal blood sample can flow first through a size-based separation module to remove enucleated cells and cellular components (e.g., analytes having a hydrodynamic size less than 6 μms) based on size. Subsequently, the enriched nucleated cells (e.g., analytes having a hydrodynamic size greater than 6 μms) white blood cells and nucleated red blood cells are treated with a reagent, such as CO_2, N_2, or $NaNO_2$, that changes the magnetic property of the red blood cells' hemoglobin. The treated sample then flows through a magnetic field (e.g., a column coupled to an external magnet), such that the paramagnetic analytes (e.g., red blood cells) will be captured by the magnetic field while the white blood cells and any other non-red blood cells will flow through the device to result in a sample enriched in nucleated red blood cells (including fetal nucleated red blood cells or fnRBC's). Additional examples of magnetic separation modules are described in US Application Serial No. 11/323,971, filed December 29, 2005 entitled "Devices and Methods for Magnetic Enrichment of Cells and Other Particles" and US Application Serial No. 11/227,904, filed September 15, 2005, entitled "Devices and Methods for Enrichment and Alteration of Cells and Other Particles".

[0079] Subsequent enrichment steps can be used to separate the rare cells (e.g. fnRBC's) from the non-rare cells maternal nucleated red blood cells. In some embodiments, a sample enriched by size-based separation followed by affinity/magnetic separation is further enriched for rare cells using fluorescence activated cell sorting (FACS) or selective lysis of a subset of the cells.

[0080] In some embodiments, enrichment involves detection and/or isolation of rare cells or rare DNA (e.g. fetal cells or fetal DNA) by selectively initiating apoptosis in the rare cells. This can be accomplished, for example, by subjecting a sample that includes rare cells (e.g. a mixed sample) to hyperbaric pressure (increased levels of CO_2; e.g. 4% CO_2). This will selectively initiate apoptosis in the rare or fragile cells in the sample (e.g. fetal cells). Once the rare cells (e.g. fetal cells) begin apoptosis, their nuclei will condense and optionally be ejected from the rare cells. At that point, the rare cells or nuclei can be detected using any technique known in the art to detect condensed nuclei, including DNA gel electrophoresis, in situ labeling of DNA nick using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP in situ nick labeling (TUNEL) (Gavrieli, Y., et al. J. Cell Biol. 119:493-501 (1992)), and ligation of DNA strand breaks having one or two-base 3' overhangs (Taq polymerase-based in situ ligation). (Didenko V., et al. J. Cell Biol. 135:1369-76 (1996)).

[0081] In some embodiments ejected nuclei can further be detected using a size based separation module adapted to selectively enrich nuclei and other analytes smaller than a predetermined size (e.g. 6 microns) and isolate them from cells and analytes having a hydrodynamic diameter larger than 6 microns. Thus, in one embodiment, the present invention contemplated detecting fetal cells/fetal DNA and optionally using such fetal DNA to diagnose or prognose a condition in a fetus. Such detection and diagnosis can occur by obtaining a blood sample from the female pregnant with the fetus, enriching the sample for cells and analytes larger than 8 microns using, for example,

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an array of obstacles adapted for size-base separation where the predetermined size of the separation is 8 microns (e.g. the gap between obstacles is up to 8 microns). Then, the enriched product is further enriched for red blood cells (RBC's) by oxidizing the sample to make the hemoglobin puramagnetic and flowing the sample through one or more magnetic regions. This selectively captures the RBC's and removes other cells (e.g. white blood cells) from the sample. Subsequently, the fnRBC's can be enriched from mnRBC's in the second enriched product by subjecting the second enriched product to hyperbaric pressure or other stimulus that selectively causes the fetal cells to begin apoptosis and condense / eject their nuclei. Such condensed nuclei are then identified/isolated using e.g. laser capture microdissection or a size based separation module that separates components smaller than 3, 4, 5 or 6 microns from a sample. Such fetal nuclei can then by analyzed using any method known in the art or described herein.

[0082] In some embodiments, when the analyte desired to be separated (e.g., red blood cells or white blood cells) is not ferromagnetic or does not have a potential magnetic property, a magnetic particle (e.g., a bead) or compound (e.g., Fe^{3+}) can be coupled to the analyte to give it a magnetic property. In some embodiments, a bead coupled to an antibody that selectively binds to an analyte of interest can be decorated with an antibody elected from the group of anti CD71 or CD75. In some embodiments a magnetic compound, such as Fe^{3+}, can be couple to an antibody such as those described above. The magnetic particles or magnetic antibodies herein may be coupled to any one or more of the devices herein prior to contact with a sample or may be mixed with the sample prior to delivery of the sample to the device(s). Magnetic particles can also be used to decorate one or more analytes (cells of interest or not of interest) to increase the size prior to performing size-based separation.

[0083] Magnetic field used to separate analytes/cells in any of the embodiments herein can uniform or non-uniform as well as external or internal to the device(s) herein. An external magnetic field is one whose source is outside a device herein (e.g., container, channel, obstacles). An internal magnetic field is one whose source is within a device contemplated herein. An example of an internal magnetic field is one where magnetic particles may be attached to obstacles present in the device (or manipulated to create obstacles) to increase surface area for analytes to interact with to increase the likelihood of binding. Analytes captured by a magnetic field can be released by demagnetizing the magnetic regions retaining the magnetic particles. For selective release of analytes from regions, the demagnetization can be limited to selected obstacles or regions. For example, the magnetic field can be designed to be electromagnetic, enabling turn-on and turn-off off the magnetic fields for each individual region or obstacle at will.

[0084] **Figure 3** illustrates an embodiment of a device configured for capture and isolation of cells expressing the transferrin receptor from a complex mixture. Monoclonal antibodies to CD71 receptor are readily available off-the-shelf and can be covalently coupled to magnetic materials comprising any conventional ferroparticles, such as, but not limited to ferrous doped polystyrene and ferroparticles or ferro-colloids (e.g., from Miltenyi and Dynal). The anti CD71 bound to magnetic particles is flowed into the device. The antibody coated particles are drawn to the obstacles (e.g., posts), floor, and walls and are retained by the strength of the magnetic field interaction between the particles and the magnetic field. The particles between the obstacles and those loosely retained with the sphere of influence of the local magnetic fields away from the obstacles are removed by a rinse.

[0085] One or more of the enrichment modules herein (e.g., size-based separation module(s) and capture module(s)) may be fluidly coupled in series or in parallel with one another. For example a first outlet from a separation module can be fluidly coupled to a capture module. In some embodiments, the separation module and capture module are integrated such that a plurality of obstacles acts both to deflect certain analytes according to size

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and direct them in a path different than the direction of analyte(s) of interest, and also as a capture module to capture, retain, or bind certain analytes based on size, affinity, magnetism or other physical property.

[0086] In any of the embodiments herein, the enrichment steps performed have a specificity and/or sensitivity greater than 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, 99.1, 99.2, 99.3, 99.4, 99.5, 99.6, 99.7, 99.8, 99.9 or 99.95%. The retention rate of the enrichment module(s) herein is such that ≥50, 60, 70, 80, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 99.9 % of the analytes or cells of interest (e.g., nucleated cells or nucleated red blood cells or nucleated from red blood cells) are retained. Simultaneously, the enrichment modules are configured to remove ≥50, 60, 70, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 99.9 % of all unwanted analytes (e.g., red blood-platelet enriched cells) from a sample.

[0087] Any of the enrichment methods herein may be further supplemented by splitting the enriched sample into aliquots or sub-samples. In some embodiments, an enriched sample is split into at least 2, 5, 10, 20, 50, 100, 200, 500, or 1000 sub-samples. Thus when an enriched sample comprises about 500 cells and is split into 500 or 1000 different sub-samples, each sub-sample will have 1 or 0 cells.

[0088] In some cases a sample is split or arranged such that each sub-sample is in a unique or distinct location (e.g. well). Such location may be addressable. Each site can further comprise a capture mechanism to capture cell(s) to the site of interest and/or release mechanism for selectively releasing cells from the cite of interest. In some cases, the well is configured to hold a single cell.

III. Sample Analysis

[0089] In some embodiments, the methods herein are used for detecting the presence or conditions of rare cells that are in a mixed sample (optionally even after enrichment) at a concentration of up to 90%, 80%, 70%, 60%, 50 %, 40%, 30%, 20%, 10%, 5% or 1% of all cells in the mixed sample, or at a concentration of less than 1:2, 1:4, 1:10, 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:5000, 1:10,000, 1:20,000, 1:50,000, 1:100,000, 1:200,000, 1:1,000,000, 1:2,000,000, 1:5,000,000, 1:10,000,000, 1:20,000,000, 1:50,000,000 or 1:100,000,000 of all cells in the sample, or at a concentration of less than 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, or 1×10^{-7} cells/μL of a fluid sample. In some embodiments, the mixed sample has a total of up to 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, or 100 rare cells (e.g. fetal cells or epithelial cells).

[0090] Enriched target cells (e.g., fnRBC) may be "binned" prior to further analysis of the enriched cells (**Figures 34 &35**). Binning is any process which results in the reduction of complexity and/or total cell number of the enriched cell output. Binning may be performed by any method known in the art or described herein. One method of binning is by serial dilution. Such dilution may be carried out using any appropriate platform (e.g., PCR wells, microtiter plates) and appropriate buffers. Other methods include nanofluidic systems which can separate samples into droplets (e.g., BioTrove, Raindance, Fluidigm). Such nanofluidic systems may result in the presence of a single cell present in a nanodroplet.

[0091] Binning may be preceded by positive selection for target cells including, but not limited to, affinity binding (e.g. using anti-CD71 antibodies). Alternately, negative selection of non-target cells may precede binning. For example, output from a size-based separation module may be passed through a magnetic hemoglobin enrichment module (MHEM) which selectively removes WBCs from the enriched sample by attracting magnetized hemoglobin-containing cells.

[0092] For example, the possible cellular content of output from enriched maternal blood which has been passed through a size-based separation module (with or without further enrichment by passing the enriched sample through a MHEM) may consist of: 1) approximately 20 fnRBC; 2) 1,500 mnRBC; 3) 4,000-40,000 WBC; 4) 15×10^6 RBC. If this sample is separated into 100 bins (PCR wells or other acceptable binning platform), each bin would be

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expected to contain: 1) 80 negative bins and 20 bins positive for one fnRBC; 2) 150 mnRBC; 3) 400-4,000 WBC; 4) $15x10^4$ RBC. If separated into 10,000 bins, each bin would be expected to contain: 1) 9,980 negative bins and 20 bins positive for one fnRBC; 2) 8,500 negative bins and 1,500 bins positive for one mnRBC; 3) <1-4 WBC; 4) $15x10^2$ RBC. One of skill in the art will recognize that the number of bins may be increased or decreased depending on experimental design and/or the platform used for binning. Reduced complexity of the binned cell populations may facilitate further genetic and/or cellular analysis of the target cells by reducing the number of non-target cells in an individual bin.

[0093] Analysis may be performed on individual bins to confirm the presence of target cells (e.g. fnRBC) in the individual bin. Such analysis may consist of any method known in the art including, but not limited to, FISH, PCR, STR detection, SNP analysis, biomarker detection, and sequence analysis **(Figures 34 &35)**.

[0094] For example, a peripheral maternal venous blood sample enriched by the methods herein can be analyzed to determine pregnancy or a condition of a fetus (e.g., sex of fetus or aneuploidy). The analysis step for fetal cells may further involves comparing the ratio of maternal to paternal genomic DNA on the identified fetal cells.

IV. Fetal Biomarkers

[0095] In some embodiments fetal biomarkers may be used to detect and/or isolate fetal cells, after enrichment or after detection of fetal abnormality or lack thereof. For example, this may be performed by distinguishing between fetal and maternal nRBCs based on relative expression of a gene (e.g., DYS1, DYZ, CD-71, ε - and ζ -globin) that is differentially expressed during fetal development. In preferred embodiments, biomarker genes are differentially expressed in the first and/or second trimester. "Differentially expressed," as applied to nucleotide sequences or polypeptide sequences in a cell or cell nuclei, refers to differences in over/under-expression of that sequence when compared to the level of expression of the same sequence in another sample, a control or a reference sample. In some embodiments, expression differences can be temporal and/or cell-specific. For example, for cell-specific expression of biomarkers, differential expression of one or more biomarkers in the cell(s) of interest can be higher or lower relative to background cell populations. Detection of such difference in expression of the biomarker may indicate the presence of a rare cell (e.g., fnRBC) versus other cells in a mixed sample (e.g., background cell populations). In other embodiments, a ratio of two or more such biomarkers that are differentially expressed can be measured and used to detect rare cells.

[0096] In one embodiment, fetal biomarkers comprise differentially expressed hemoglobins. Erythroblasts (nRBCs) are very abundant in the early fetal circulation, virtually absent in normal adult blood and by having a short finite lifespan, there is no risk of obtaining fnRBC which may persist from a previous pregnancy. Furthermore, unlike trophoblast cells, fetal erythroblasts are not prone to mosaic characteristics.

[0097] Yolk sac erythroblasts synthesize ε -, ζ -,γ- and α -globins, these combine to form the embryonic hemoglobins. Between six and eight weeks, the primary site of erythropoiesis shifts from the yolk sac to the liver, the three embryonic hemoglobins are replaced by fetal hemoglobin (HbF) as the predominant oxygen transport system, and ε - and ζ -globin production gives way to γ -, α - and β -globin production within definitive erythrocytes (Peschle et al., 1985). HbF remains the principal hemoglobin until birth, when the second globin switch occurs and β -globin production accelerates.

[0098] Hemoglobin (Hb) is a heterodimer composed of two identical α globin chains and two copies of a second globin. Due to differential gene expression during fetal development, the composition of the second chain changes from ε globin during early embryonic development (1 to 4 weeks of gestation) to γ globin during fetal development (6 to 8 weeks of gestation) to β globin in neonates and adults as illustrated in (Table 1).

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[0099] Table 1. Relative expression of ε, γ and β in maternal and fetal RBCs.

		ε	γ	B
1st trimester	Fetal	++	++	-
	Maternal	-	+/-	++
2nd trimester	Fetal	-	++	+/-
	Maternal	-	+/-	++

[00100] In the late-first trimester, the earliest time that fetal cells may be sampled by CVS, fnRBCs contain, in addition to α globin, primarily ε and γ globin. In the early to mid second trimester, when amniocentesis is typically performed, fnRBCs contain primarily γ globin with some adult β globin. Maternal cells contain almost exclusively α and β globin, with traces of γ detectable in some samples. Therefore, by measuring the relative expression of the ε, γ and β genes in RBCs purified from maternal blood samples, the presence of fetal cells in the sample can be determined. Furthermore, positive controls can be utilized to assess failure of the FISH analysis itself.

[00101] In various embodiments, fetal cells are distinguished from maternal cells based on the differential expression of hemoglobins β, γ or ε. Expression levels or RNA levels can be determined in the cytoplasm or in the nucleus of cells. Thus in some embodiments, the methods herein involve determining levels of messenger RNA (mRNA), ribosomal RNA (rRNA), or nuclear RNA (nRNA).

[00102] In some embodiments, identification of fnRBCs can be achieved by measuring the levels of at least two hemoglobins in the cytoplasm or nucleus of a cell. In various embodiments, identification and assay is from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 or 20 fetal nuclei. Furthermore, total nuclei arrayed on one or more slides can number from about 100, 200, 300, 400, 500, 700, 800, 5000, 10,000, 100,000, 1,000,000, 2,000,000 to about 3,000,000. In some embodiments, a ratio for γ/β or ε/β is used to determine the presence of fetal cells, where a number less than one indicates that a fnRBC(s) is not present. In some embodiments, the relative expression of γ/β or ε/β provides a fnRBC index ("FNI"), as measured by γ or ε relative to β. In some embodiments, a FNI for γ/β greater than 5, 10, 15, 20, 25, 30, 35, 40, 45, 90, 180, 360, 720, 975, 1020, 1024, 1250 to about 1250, indicate that a fnRBC(s) is present. In yet other embodiments, a FNI for γ/β of less than about 1 indicates that a fnRBC(s) is not present. Preferably, the above FNI is determined from a sample obtained during a first trimester. However, similar ratios can be used during second trimester and third trimester.

[00103] In some embodiments, the expression levels are determined by measuring nuclear RNA transcripts including, nascent or unprocessed transcripts. In another embodiment, expression levels are determined by measuring mRNA, including ribosomal RNA. There are many methods known in the art for imaging (e.g., measuring) nucleic acids or RNA including, but not limited to, using expression arrays from Affymetrix, Inc. or Illumina, Inc.

[00104] RT-PCR primers can be designed by targeting the globin variable regions, selecting the amplicon size, and adjusting the primers annealing temperature to achieve equal PCR amplification efficiency. Thus TaqMan probes can be designed for each of the amplicons with well-separated fluorescent dyes, Alexa fluor®-355 for ε, Alexa Fluor®-488 for γ, and Alexa Fluor-555 for β. The specificity of these primers can be first verified using ε, γ, and β cDNA as templates. The primer sets that give the best specificity can be selected for further assay development. As an alternative, the primers can be selected from two exons spanning an intron sequence to amplify only the mRNA to eliminate the genomic DNA contamination.

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[00105] The primers selected can be tested first in a duplex format to verify their specificity, limit of detection, and amplification efficiency using target cDNA templates. The best combinations of primers can be further tested in a triplex format for its amplification efficiency, detection dynamic range, and limit of detection.

[00106] Various commercially available reagents are available for RT-PCR, such as One-step RT-PCR reagents, including Qiagen One-Step RT-PCR Kit and Applied Biosytems TaqMan One-Step RT-PCR Master Mix Reagents kit. Such reagents can be used to establish the expression ratio of ε, γ, *and* β using purified RNA from enriched samples. Forward primers can be labeled for each of the targets, using Alexa fluor-355 for ε, Alexa fluor-488 for γ, and Alexa fluor-555 for β. Enriched cells can be deposited by cytospinning onto glass slides. Additionally, cytospinning the enriched cells can be performed after in situ RT-PCR. Thereafter, the presence of the fluorescent-labeled amplicons can be visualized by fluorescence microscopy. The reverse transcription time and PCR cycles can be optimized to maximize the amplicon signal:background ratio to have maximal separation of fetal over maternal signature. Preferably, signal:background ratio is greater than 5, 10, 50 or 100 and the overall cell loss during the process is less than 50, 10 or 5%.

V. Fetal Cell Analysis

[00107] **Figure 4** illustrates an overview of some embodiments of the present invention.

[00108] Aneuploidy means the condition of having less than or more than the normal diploid number of chromosomes. In other words, it is any deviation from euploidy. Aneuploidy includes conditions such as monosomy (the presence of only one chromosome of a pair in a cell's nucleus), trisomy (having three chromosomes of a particular type in a cell's nucleus), tetrasomy (having four chromosomes of a particular type in a cell's nucleus), pentasomy (having five chromosomes of a particular type in a cell's nucleus), triploidy (having three of every chromosome in a cell's nucleus), and tetraploidy (having four of every chromosome in a cell's nucleus). Birth of a live triploid is extraordinarily rare and such individuals are quite abnormal, however triploidy occurs in about 2-3% of all human pregnancies and appears to be a factor in about 15% of all miscarriages. Tetraploidy occurs in approximately 8% of all miscarriages. (http://www.emedicine.com/med/topic3241.htm).

[00109] In step **400**, a sample is obtained from an animal, such as a human. In some embodiments, animal or human is pregnant, suspected of being pregnant, or may have been pregnant, and, the systems and methods herein are used to diagnose pregnancy and/or conditions of the fetus (e.g. trisomy). In some embodiments, the animal or human is suspected of having a condition, has a condition, or had a condition (e.g., cancer) and, the systems and methods herein are used to diagnose the condition, determine appropriate therapy, and/or monitor for recurrence.

[00110] In both scenarios a sample obtained from the animal can be a blood sample e.g., of up to 50, 40, 30, 20, or 15 mL. In some cases multiple samples are obtained from the same animal at different points in time (e.g. before therapy, during therapy, and after therapy, or during 1st trimester, 2nd trimester, and 3rd trimester of pregnancy).

[00111] In optional step **402**, rare cells (e.g., fetal cells or epithelial cells) or DNA of such rare cells are enriched using one or more methods known in the art or described herein. For example, to enrich fetal cells from a maternal blood sample, the sample can be applied to a size-base separation module (e.g., two-dimensional array of obstacles) configured to direct cells or particles in the sample greater than 8 microns to a first outlet and cells or particles in the sample smaller than 8 microns to a second outlet. The fetal cells can subsequently be further enriched from maternal white blood cells (which are also greater than 8 microns) based on their potential magnetic property. For example, N_2 or anti-CD71 coated magnetic beads is added to the first enriched product to make the hemoglobin in the red blood cells (maternal and fetal) paramagnetic. The enriched sample is then flowed through a column coupled to an external magnet. This captures both the fnRBC's and mnRBC's creating a second enriched product. The sample can then be subjected to hyperbaric pressure or other stimulus to initiate apoptosis in the fetal cells. Fetal

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cells/nuclei can then be enriched using microdissection, for example. It should be noted that even an enriched product can be dominated (>50%) by cells not of interest (e.g. maternal red blood cells). In some cases an enriched sample has the rare cells (or rare genomes) consisting of up to 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, or 50% of all cells (or genomes) in the enriched sample. For example, using the systems herein, a maternal blood sample of 20 mL from a pregnant human can be enriched for fetal cells such that the enriched sample has a total of about 500 cells, 2% of which are fetal and the rest are maternal.

[00112] In step **404**, the enriched product is split between two or more discrete locations. In some embodiments, a sample is split into at least 2, 10, 20, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3,000, 4,000, 5000, or 10,000 total different discrete sites or about 100, 200, 500, 1000, 1200, 1500 sites. In some embodiments, output from an enrichment module is serially divided into wells of a 1536 microwell plate (Figure 8). This can result in one cell or genome per location or 0 or 1 cell or genome per location. In some embodiments, cell splitting results in more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 200, 500, 1000, 2000, 5000, 10,000, 20,000, 50,000, 100,000, 200,000, or 500,000 cells or genomes per location. When splitting a sample enriched for epithelial cells, endothelial cells, or CTC's, the load at each discrete location (e.g., well) can include several leukocytes, while one only some of the loads includes one or more CTC's. When splitting a sample enriched for fetal cells preferably each site includes 0 or 1 fetal cells.

[00113] Examples of discrete locations which could be used as addressable locations include, but are not limited to, wells, bins, sieves, pores, geometric sites, slides, matrixes, membranes, electric traps, gaps, obstacles, or in-situ within a cell or nuclear membrane. In some embodiments, the discrete cells are addressable such that one can correlate a cell or cell sample with a particular location.

[00114] Examples of methods for splitting a sample into discrete addressable locations include, but are not limited to, fluorescent activated cell sorting (FACS) (Sherlock, JV et al. Ann. Hum. Genet. 62 (Pt. 1): 9-23 (1998)), micromanipulation (Samura, O., Ct al Hum. Genet. 107(1):28-32 (2000)) and dilution strategies (Findlay, I. et al. Mol. Cell. Endocrinol. 183 Suppl 1: S5-12 (2001)). Other methods for sample splitting cell sorting and splitting methods known in the art may also be used. For example, samples can be split by affinity sorting techniques using affinity agents (e.g. antibodies) bound to any immobilized or mobilized substrate (Samura O., et al., Hum. Genet. 107(1):28-32 (2000)). Such affinity agents can be specific to a cell type e.g. RBC's fetal cells epithelial cells including those specifically binding EpCAM, antigen-i, or CD-71.

[00115] In some embodiments, a sample or enriched sample is transferred to a cell sorting device that includes an array of discrete locations for capturing cells traveling along a fluid flow. The discrete locations can be arranged in a defined pattern across a surface such that the discrete sites are also addressable. In some embodiments, the sorting device is coupled to any of the enrichment devices known in the art or disclosed herein. Examples of cell sorting devices included are described in International Publication No. WO 01/35071. Examples of surfaces that may be used for creating arrays of cells in discrete addressable sites include, but are not limited to, cellulose, cellulose acetate, nitrocellulose, glass, quartz or other crystalline substrates such as gallium arsenide, silicones, metals, semiconductors, various plastics and plastic copolymers, cyclo-olefin polymers, various membranes and gels, microspheres, beads and paramagnetic or supramagnetic microparticles.

[00116] In some embodiments, a sorting device comprises an array of wells or discrete locations wherein each well or discrete location is configured to hold up to 1 cell. Each well or discrete addressable location may have a capture mechanism adapted for retention of such cell (e.g. gravity, suction, etc.) and optionally a release mechanism for selectively releasing a cell of interest from a specific well or site (e.g. bubble actuation). Figure B illustrates such an embodiment.

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[00117] In step **406**, nucleic acids of interest from each cell or nuclei arrayed are tagged by amplification. Preferably, the amplified/tagged nucleic acids include at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 90, 90 or 100 polymorphic genomic DNA regions such as short tandem repeats (STRs) or variable number of tandem repeats ("VNTR"). When the amplified DNA regions include one or more STR/s/, the STR/s/ are selected for high heterozygosity (variety of alleles) such that the paternal allele of any fetal cell is more likely to be distinct in length from the maternal allele. This results in improved power to detect the presence of fetal cells in a mixed sample and any potential of fetal abnormalities in such cells. In some embodiment, STR(s) amplified are selected for their association with a particular condition. For example, to determine fetal abnormality an STR sequence comprising a mutation associated with fetal abnormality or condition is amplified. Examples of STRs that can be amplified/analyzed by the methods herein include, but are not limited to D21S1414, D21S1411, D21S1412, D21S11 MBP, D13S634, D13S631, D18S535, AmgXY and XHPRT. Additional STRs that can be amplified/analyzed by the methods herein include, but are not limited to, those at locus F13B (1:q31-q32); TPOX (2:p23-2pter); FIBRA (FGA) (4:q28); CSF1PO (5:q33.3-q34); FI3A (6:p24-p25); THOI (11:p15-15.5); VWA (12:p12-pter); CDU (12p12-pter); D14S1434 (14:q32.13); CYAR04 (p450) (15:q21.1) D21S11 (21:q11-q21) and D22S1045 (22:q12.3). In some cases, STR loci are chosen on a chromosome suspected of trisomy and on a control chromosome. Examples of chromosomes that are often trisomic include chromosomes 21, 18, 13, and X. In some cases, 1 or more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 STRs are amplified per chromosome tested (Samura, O. et al., Clin. Chem. 47(9):1622-6 (2001)). For example amplification can be used to generate amplicons of up to 20, up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 150, up to 200, up to 300, up to 400, up to 500 or up to 1000 nucleotides in length. Di-, tri-, tetra- , or penta-nucleotide repeat STR loci can be used in the methods described herein.

[00118] To amplify and tag genomic DNA region(s) of interest, PCR primers can include: (i) a primer element, (ii) a sequencing element, and (iii) a locator element.

[00119] The primer element is configured to amplify the genomic DNA region of interest (e.g. STR). The primer element includes, when necessary, the upstream and downstream primers for the amplification reactions. Primer elements can be chosen which are multiplexible with other primer pairs from other tags in the same amplification reaction (e.g. fairly uniform melting temperature, absence of cross-priming on the human genome, and absence of primer-primer interaction based on sequence analysis). The primer element can have at least 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40 or 50 nucleotide bases, which are designed to specifically hybridize with and amplify the genomic DNA region of interest.

[00120] The sequencing element can be located on the 5' end of each primer element or nucleic acid tag. The sequencing element is adapted to cloning and/or sequencing of the amplicons. (Marguiles, M, Nature 437 (7057): 376-80) The sequencing element can be about 4, 6, 8, 10, 18, 20, 28, 36, 46 or 50 nucleotide bases in length.

[00121] The locator element (also known as a unique tag sequence), which is often incorporated into the middle part of the upstream primer, can include a short DNA or nucleic acid sequence between 4-20 bp in length (e.g., about 4, 6, 8, 10, or 20 nucleotide bases). The locator element makes it possible to pool the amplicons from all discrete addressable locations following the amplification step and analyze the amplicons in parallel. In some embodiments each locator element is specific for a single addressable location.

[00122] Tags are added to the cells/DNA at each discrete location using an amplification reaction. Amplification can be performed using PCR or by a variety of methods including, but not limited to, singleplex PCR, quantitative PCR, quantitative fluorescent PCR (QF-PCR), multiplex fluorescent PCR (MF-PCR), real time PCR (RT-PCR), single cell PCR, restriction fragment length polymorphism PCR (PCR-RFLP), PCR-RFLP/RT-PCR-RFLP, hot start

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PCR, nested PCR, in situ polonony PCR, in situ rolling circle amplification (RCA), bridge PCR, picotiter PCR, multiple strand displacement amplification (MDA), and emulsion PCR. Other suitable amplification methods include the ligase chain reaction (LCR), transcription amplification, self-sustained sequence replication, selective amplification of target polynucleotide sequences, consensus sequence primed polymerase chain reaction (CP-PCR), arbitrarily primed polymerase chain reaction (AP-PCR), degenerate oligonucleotide-primed PCR (DOP-PCR) and nucleic acid based sequence amplification (NABSA). Additional examples of amplification techniques using PCR primers are described in, U.S. Pat. Nos. 5,242,794, 5,494,810, 4,988,617 and 6,582,938.

[00123] In some embodiments, a further PCR amplification is performed using nested primers for the one or more genomic DNA regions of interest to ensure optimal performance of the multiplex amplification. The nested PCR amplification generates sufficient genomic DNA starting material for further analysis such as in the parallel sequencing procedures below.

[00124] In step **408**, genomic DNA regions tagged/amplified are pooled and purified prior to further processing. Methods for pooling and purifying genomic DNA are known in the art.

[00125] In step **410**, pooled genomic DNA/amplicons are analyzed to measure, e.g. allele abundance of genomic DNA regions (e.g. STRs amplified). In some embodiments such analysis involves the use of capillary gel electrophoresis (CGE). In other embodiments, such analysis involves sequencing or ultra deep sequencing.

[00126] Sequencing can be performed using the classic Sanger sequencing method or any other method known in the art.

[00127] For example, sequencing can occur by sequencing-by-synthesis, which involves inferring the sequence of the template by synthesizing a strand complementary to the target nucleic acid sequence. Sequence-by-synthesis can be initiated using sequencing primers complementary to the sequencing element on the nucleic acid tags. The method involves detecting the identity of each nucleotide immediately after (substantially real-time) or upon (real-time) the incorporation of a labeled nucleotide or nucleotide analog into a growing strand of a complementary nucleic acid sequence in a polymerase reaction. After the successful incorporation of a label nucleotide, a signal is measured and then nulled by methods known in the art. Examples of sequence-by-synthesis methods are described in U.S. Application Publication Nos. 2003/0044781, 2006/0024711, 2006/0024678 and 2005/0100932. Examples of labels that can be used to label nucleotide or nucleotide analogs for sequencing-by-synthesis include, but are not limited to, chromophores, fluorescent moieties, enzymes, antigens, heavy metal, magnetic probes, dyes, phosphorescent groups, radioactive materials, chemiluminescent moeities, scattering or fluorescent nanoparticles, Raman signal generating moieties, and electrochemical detection moieties. Sequencing-by-synthesis can generate at least 1,000, at least 5,000, at least 10,000, at least 20,000, 30,000, at least 40,000, at least 50,000, at least 100,000 or at least 500,000 reads per hour. Such reads can have at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 120 or at least 150 bases per read.

[00128] Another sequencing method involves hybridizing the amplified genomic region of interest to a primer complementary to it. This hybridization complex is incubated with a polymerase, ATP sulfurylase, luciferase, apyrase, and the substrates luciferin and adenosine 5' phosphosulfate. Next, deoxynucleotide triphosphates corresponding to the bases A, C, G, and T (U) are added sequentially. Each base incorporation is accompanied by release of pyrophosphate, converted to ATP by sulfurylase, which drives synthesis of oxyluciferin and the release of visible light. Since pyrophosphate release is equimolar with the number of incorporated bases, the light given off is proportional to the number of nucleotides adding in any one step. The process is repeated until the entire sequence is determined.

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[00129] Yet another sequencing method involves a four-color sequencing by ligation scheme (degenerate ligation), which involves hybridizing an anchor primer to one of four positions. Then an enzymatic ligation reaction of the anchor primer to a population of degenerate nonamers that are labeled with fluorescent dyes is performed. At any given cycle, the population of nonamers that is used is structure such that the identity of one of its positions is correlated with the identity of the fluorophore attached to that nonamer. To the extent that the ligase discriminates for complementarily at that queried position, the fluorescent signal allows the inference of the identity of the base. After performing the ligation and four-color imaging, the anchor primer:nonamer complexes are stripped and a new cycle begins. Methods to image sequence information after performing ligation are known in the art.

[00130] Preferably, analysis involves the use of ultra-deep sequencing, such as described in Marguiles et al., Nature 437 (7057): 376-80 (2005). Briefly, the amplicons are diluted and mixed with beads such that each bead captures a single molecule of the amplified material. The DNA molecule on each bead is then amplified to generate millions of copies of the sequence which all remain bound to the bead. Such amplification can occur by PCR. Each bead can be placed in a separate well, which can be a (optionally addressable) picolitre-sized well. In some embodiments, each bead is captured within a droplet of a PCR-reaction-mixture-in-oil-emulsion and PCR amplification occurs within each droplet. The amplification on the bead results in each bead carrying at least one million, at least 5 million, or at least 10 million copies of the original amplicon coupled to it. Finally, the beads are placed into a highly parallel sequencing by synthesis machine which generates over 400,000 reads (~100bp per read) in a single 4 hour run.

[00131] Other methods for ultra-deep sequencing that can be used are described in Hong, S. et al. Nat. Biotechnol. 22(4):435-9 (2004); Bennett, B. et al. Pharmacogenomics 6(4):373-82 (2005); Shendure, P. et al. Science 309 (5741):1728-32 (2005).

[00132] The role of the ultra-deep sequencing is to provide an accurate and quantitative way to measure the allele abundances for each of the STRs. The total required number of reads for each of the aliquot wells is determined by the number of STRs, the error rates of the multiplex PCR, and the Poisson sampling statistics associated with the sequencing procedures.

[00133] In one example, the enrichment output from step **402** results in approximately 500 cells of which 98% are maternal cells and 2% are fetal cells. Such enriched cells are subsequently split into 500 discrete locations (e.g., wells) in a microtiter plate such that each well contains 1 cell. PCR is used to amplify STR's (~3-10 STR loci) on each chromosome of interest. Based on the above example, as the fetal/maternal ratio goes down, the aneuploidy signal becomes diluted and more loci are needed to average out measurement errors associated with variable DNA amplification efficiencies from locus to locus. The sample division into wells containing ~1 cell proposed in the methods described herein achieves pure or highly enriched fetal/maternal ratios in some wells, alleviating the requirements for averaging of PCR errors over many loci.

[00134] In one example, let 'f' be the fetal/maternal DNA copy ratio in a particular PCR reaction. Trisomy increases the ratio of maternal to paternal alleles by a factor 1+f/2. PCR efficiencies vary from allele to allele within a locus by a mean square error in the logarithm given by σ_{allele}^2, and vary from locus to locus by σ_{locus}^2, where this second variance is apt to be larger due to differences in primer efficiency. N_a is the loci per suspected aneuploid chromosome and N_c is the control loci. If the mean of the two maternal allele strengths at any locus is 'm' and the paternal allele strength is 'p,' then the squared error expected is the mean of the ln(ratio(m/p)), where this mean is taken over N loci is given by $2(\sigma_{allele}^2)/N$. When taking the difference of this mean of ln(ratio(m/p)) between a suspected aneuploidy region and a control region, the error in the difference is given by

$$\sigma_{diff}^2 = 2(\sigma_{allele}^2)/N_a + 2(\sigma_{allele}^2)/N_c \quad (1)$$

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[00135] For a robust detection of aneuploidy we require

$$3\sigma_{diff} < f/2.$$

[00136] For simplicity, assuming $N_a = N_c = N$ in Equation 1, this gives the requirement

$$6\sigma_{allele}/N^{1/2} < f/2, \quad (3)$$

or a minimum N of

$$N = 144(\sigma_{allele}/f)^2 \quad (4)$$

[00137] In the context of trisomy detection, the suspected aneuploidy region is usually the entire chromosome and N denotes the number of loci per chromosome. For reference, Equation 3 is evaluated for N in the following **Table 2** for various values of σ_{allele} and f.

		f	
σ_{allele}	**0.1**	**0.3**	**1.0**
0.1	144	16	1
0.3	1296	144	13
1.0	14400	1600	144

Table 2. Required number of loci per chromosome as a function of σ_{allele} and f.

Since sample splitting decreases the number of starting genome copies which increases σ_{allele} at the same time that it increases the value of f in some wells, the methods herein are based on the assumption that the overall effect of splitting is favorable; i.e., that the PCR errors do not increase too fast with decreasing starting number of genome copies to offset the benefit of having some wells with large f. The required number of loci can be somewhat larger because for many loci the paternal allele is not distinct from the maternal alleles, and this incidence depends on the heterozygosity of the loci. In the case of highly polymorphic STRs, this amounts to an approximate doubling of N.

[00138] The role of the sequencing is to measure the allele abundances output from the amplification step. It is desirable to do this without adding significantly more error due to the Poisson statistics of selecting only a finite number of amplicons for sequencing. The rms error in the ln(abundance) due to Poisson statistics is approximately $(N_{reads})^{-1/2}$. It is desirable to keep this value less than or equal to the PCR error σ_{allele}. Thus, a typical paternal allele needs to be allocated at least $(\sigma_{allele})^{-2}$ reads. The maternal alleles, being more abundant, do not add appreciably to this error when forming the ratio estimate for m/p. The mixture input to sequencing contains amplicons from N_{loci} loci of which roughly an abundance fraction f/2 are paternal alleles. Thus, the total required number of reads for each of the aliquot wells is given approximately by $2N_{loci}/(f\,\sigma_{allele}^2)$. Combining this result with Equation 4, it is found a total number of reads over all the wells given approximately by

$$N_{reads} = 288\, N_{wells}\, f^3. \quad (5)$$

[00139] When performing sample splitting, a rough approximation is to stipulate that the sample splitting causes f to approach unity in at least a few wells. If the sample splitting is to have advantages, then it must be these wells which dominate the information content in the final result. Therefore, Equation (5) with f=1 is adopted, which suggests a minimum of about 300 reads per well. For 500 wells, this gives a minimum requirement for ~150,000 sequence reads. Allowing for the limited heterozygosity of the loci tends to increase the requirements (by a factor of ~2 in the case of STRs), while the effect of reinforcement of data from multiple wells tends to relax the requirements with respect to this result (in the baseline case examined above it is assumed that ~10 wells have a pure fetal cell). Thus the required total number of reads per patient is expected to be in the range 100,000 – 300,000.

[00140] In step **412**, wells with rare cells/alleles (e.g., fetal alleles) are identified. The locator elements of each tag can be used to sort the reads (~200,000 sequence reads) into 'bins' which correspond to the individual wells of the

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microtiter plates (~500 bins). The sequence reads from each of the bins (~400 reads per bin) are then separated into the different genomic DNA region groups, (e.g. STR loci,) using standard sequence alignment algorithms. The aligned sequences from each of the bins are used to identify rare (e.g., non-maternal) alleles. It is estimated that on average a 15 ml blood sample from a pregnant human will result in ~10 bins having a single fetal cell each.

[00141] The following are two examples by which rare alleles can be identified. In a first approach, an independent blood sample fraction known to contain only maternal cells can be analyzed as described above in order to obtain maternal alleles. This sample can be a white blood cell fraction or simply a dilution of the original sample before enrichment. In a second approach, the sequences or genotypes for all the wells can be similarity-clustered to identify the dominant pattern associated with maternal cells. In either approach, the detection of non-maternal alleles determines which discrete location (e.g. well) contained fetal cells. Determining the number of bins with non-maternal alleles relative to the total number of bins provides an estimate of the number of fetal cells that were present in the original cell population or enriched sample. Bins containing fetal cells are identified with high levels of confidence because the non-maternal alleles are detected by multiple independent polymorphic DNA regions, e.g. STR loci.

[00142] In step **414**, condition of rare cells or DNA is determined. This can be accomplished by determining abundance of selected alleles (polymorphic genomic DNA regions) in bin(s) with rare cells/DNA. In some embodiments, allele abundance is used to determine aneuploidy, e.g. chromosomes 13, 18 and 21. Abundance of alleles can be determined by comparing ratio of maternal to paternal alleles for each genomic region amplified (e.g., ~12 STR's). For example, if 12 STRs are analyzed, for each bin there are 33 sequence reads for each of the STRs. In a normal fetus, a given STR will have 1:1 ratio of the maternal to paternal alleles with approximately 16 sequence reads corresponding to each allele (normal diallelic). In a trisomic fetus, three doses of an STR marker will be detected either as three alleles with a 1:1:1 ratio (trisomic triallelic) or two alleles with a ratio of 2:1 (trisomic diallelic). (Adinolfi, P. et al., Prenat. Diagn, 17(13):1299-311 (1997)). In rare instances all three alleles may coincide and the locus will not be informative for that individual patient. In some embodiments, the information from the different DNA regions on each chromosome are combined to increase the confidence of a given aneuploidy call. In some embodiments, the information from the independent bins containing fetal cells can also be combined to further increase the confidence of the call.

[00143] In some embodiments allele abundance is used to determine segmental anuepolidy. Normal diploid cells have two copies of each chromosome and thus two alleles of each gene or loci. Changes in the allele abundance for a particular chromosomal region may be indicative of a chromosomal rearrangement, such as a deletion, duplication or translocation event. In some embodiments, the information from the different DNA regions on each chromosome are combined to increase the confidence of a given segmental aneuploidy call. In some embodiments, the information from the independent bins containing fetal cells can also be combined to further increase the confidence of the call.

[00144] The determination of fetal trisomy can be used to diagnose conditions such as abnormal fetal genotypes, including, trisomy 13, trisomy 18, trisomy 21 (Down syndrome) and Klinefelter Syndrome (XXY). Other examples of abnormal fetal genotypes include, but are not limited to, aneuploidy such as, monosomy of one or more chromosomes (X chromosome monosomy, also known as Turner's syndrome), trisomy of one or more chromosomes (13, 18, 21, and X), tetrasomy and pentasomy of one or more chromosomes (which in humans is most commonly observed in the sex chromosomes, e.g. XXXX, XXYY, XXXY, XYYY, XXXXX, XXXXY, XXXYY, XYYYY and XXYYY), triploidy (three of every chromosome, e.g. 69 chromosomes in humans), tetraploidy (four of every chromosome, e.g. 92 chromosomes in humans) and multiploidy. In some embodiments, an abnormal fetal genotype

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is a segmental aneuploidy. Examples of segmental aneuploidy include, but are not limited to, 1p36 duplication, dup(17)(p11.2p11.2) syndrome, Down syndrome, Pelizaeus-Merzbacher disease, dup(22)(q11.2q11.2) syndrome, and cat-eye syndrome. In some cases, an abnormal fetal genotype is due to one or more deletions of sex or autosomal chromosomes, which may result in a condition such as Cri-du-chat syndrome, Wolf-Hirschhorn, Williams-Beuren syndrome, Charcot-Marie-Tooth disease, Hereditary neuropathy with liability to pressure palsies, Smith-Magenis syndrome, Neurofibromatosis, Alagille syndrome, Velocardiofacial syndrome, DiGeorge syndrome, Steroid sulfatase deficiency, Kallmann syndrome, Microphthalmia with linear skin defects, Adrenal hypoplasia, Glycerol kinase deficiency, Pelizaeus-Merzbacher disease, Testis-determining factor on Y, Azospermia (factor a), Azospermia (factor b), Azospermia (factor c), or 1p36 deletion. In some embodiments, a decrease in chromosomal number results in an XO syndrome.

[00145] In one embodiment, the methods of the invention allow for the determination of maternal or paternal trisomy. In some embodiments, the methods of the invention allow for the determination of trisomy or other conditions in fetal cells in a mixed maternal sample arising from more than one fetus.

[00146] In another aspect of the invention, standard quantitative genotyping technology is used to declare the presence of fetal cells and to determine the copy numbers (ploidies) of the fetal chromosomes. Several groups have demonstrated that quantitative genotyping approaches can be used to detect copy number changes (Wang, Moorhead et al. 2005). However, these approaches do not perform well on mixtures of cells and typically require a relatively large number of input cells (~10,000). The current invention addresses the complexity issue by performing the quantitative genotyping reactions on individual cells. In addition, multiplex PCR and DNA tags are used to perform the thousands of genotyping reaction on single cells in highly parallel fashion.

[00147] An overview of this embodiment is illustrated in **Figure 5**.

[00148] In step **500**, a sample (e.g., a mixed sample of rare and non-rare cells) is obtained from an animal or a human. See, e.g., step **400** of Figure 4. Preferably, the sample is a peripheral maternal blood sample.

[00149] In step **502**, the sample is enriched for rare cells (e.g., fetal cells) by any method known in the art or described herein. See, e.g., step **402** of **Figure 4**.

[00150] In step **504**, the enriched product is split into multiple distinct sites (e.g., wells). See, e.g., step 404 of Figure 4.

[00151] In step **506**, PCR primer pairs for amplifying multiple (e.g., 2 – 100) highly polymorphic genomic DNA regions (e.g., SNPs) are added to each discrete site or well in the array or microtiter plate. For example, PCR primer pairs for amplifying SNPs along chromosome 13, 18, 21 and/or X can be designed to detect the most frequent aneuoploidies. Other PCR primer pairs can be designed to amplify SNPs along control regions of the genome where aneuploidy is not expected. The genomic loci (e.g., SNPs) in the aneuploidy region or aneuploidy suspect region are selected for high polymorphism such that the paternal alleles of the fetal cells are more likely to be distinct from the maternal alleles. This improves the power to detect the presence of fetal cells in a mixed sample as well as fetal conditions or abnormalities. SNPs can also be selected for their association with a particular condition to be detected in a fetus. In some cases, one or more than one, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 SNPs are analyzed per target chromosome (e.g., 13, 18, 21, and/or X). The increase number of SNPs interrogated per chromosome ensures accurate results. PCR primers are chosen to be multiplexible with other pairs (fairly uniform melting temperature, absence of cross-priming on the human genome, and absence of primer-primer interaction based on sequence analysis). The primers are designed to generate amplicons 10-200, 20-180, 40-160, 60-140 or 70-100 bp in size to increase the performance of the multiplex PCR.

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[00152] A second of round of PCR using nested primers may be performed to ensure optimal performance of the multiplex amplification. The multiplex amplification of single cells is helpful to generate sufficient starting material for the parallel genotyping procedure. Multiplex PCT can be performed on single cells with minimal levels of allele dropout and preferential amplification. See Sherlock, J., et al. Ann. Hum. Genet. 61 (Pt 1): 9-23 (1998); and Findlay, I., et al. Mol. Cell. Endocrinol. 183 Suppl. 1: S5-12 (2001).

[00153] In step **508**, amplified polymorphic DNA region(s) of interest (e.g., SNPs) are tagged e.g., with nucleic acid tags. Preferably, the nucleic acid tags serve two roles: to determine the identity of the different SNPs and to determine the identity of the bin from which the genotype was derived. Nucleic acid tags can comprise primers that allow for allele-specific amplification and/or detection. The nucleic acid tags can be of a variety of sizes including up to 10 base pairs, 10-40, 15-30, 18-25 or ~22 base pair long.

[00154] In some embodiments, a nucleic acid tag comprises a molecular inversion probe (MIP). Examples of MIPs and their uses are described in Hardenbol, P., et al., Nat. Biotechnol. 21(6):673-8 (2003); Hardenbol, P., et al., Genome Res. 15(2):269-75 (2005); and Wang, Y., et al., Nucleic Acids Res. 33(21):e183 (2005). **Figure 7A** illustrates one example of a MIP assay used herein. The MIP tag can include a locator element to determine the identity of the bin from which the genotype was derived. For example, when output from an enrichment procedure results in about 500 cells, the enriched product / cells can be split into a microliter plate containing 500 wells such that each cell is in a different distinct well. **Figure 7B** illustrates a microtiter plate with 500 wells each of which contains a single cell. Each cell is interrogated at 10 different SNPs per chromosome, on 4 chromosomes (e.g., chromosomes 13, 18, 21 and X). This analysis requires 40 MIPs per cell / well for a total of 20,000 tags per 500 wells (i.e., 4 chromosomes x 10 SNPs x 500 wells). The tagging step can also include amplification of the MIPs after their rearrangement or enzymatic "gap fill".

[00155] In one embodiment, a nucleic acid tag comprises a unique property, such as a difference in mass or chemical properties from other tags. In another embodiments a nucleic acid tag comprises a photoactivatable label, so that it crosslinks where it binds. In another embodiment a nucleic acid tag can be used as a linker for ultra deep sequencing. In another embodiment a nucleic acid tag can be used as a linker for arrays. In another embodiment a nucleic acid tag comprises a unique fluorescent label, (Such as FAM, JOE, ROX, NED, HEX, SYBR, PET, TAMRA, VIC, CY-3, CY-5, dR6G, DS-33, LIZ, DS-02, dR110, and Texas Red) which can be used to differentiate individual DNA fragments. In another embodiment a nucleic acid tag can serve as primer or hybridization site for a probe, to facilitate signal amplification or detection from a single cell by using a tractable marker. In some embodiments the labeled nucleic acid tag can be analyzed using a system coupled to a light source, such as an ABI 377, 310, 3700 or any other system which can detect fluorescently labeled DNA.

[00156] In step **510**, the tagged amplicons are pooled together for further analysis.

[00157] In step **512**, the genotype at each polymorphic site is determined and/or quantified using any technique known in the art. In one embodiment, genotyping occurs by hybridization of the MIP tags to a microarray containing probes complementary to the sequences of each MIP tag. See US Patent Nos. 6,858,412.

[00158] Using the example described above with the MIP probes, the 20,000 tags are hybridized to a single tag array containing complementary sequences to each of the tagged MIP probes. Microarrays (e.g. tag arrays) can include a plurality of nucleic acid probes immobilized to discrete spots (e.g., defined locations or assigned positions) on a substrate surface. For example, a microarray can have at least 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 500, 1,000, 5,000, 10,000, 15,000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, or 100,000 different probes complementary to MIP tagged probes. Methods to prepare microarrays capable to monitor several genes according to the methods of the invention are well known in the art. Examples of microarrays that can be used in

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nucleic acid analysis that may be used are described in US Pat 6,300,063, US Pat 5,837,832, US Pat 6,969, 589, US Pat 6,040,138, US Pat 6,858, 412, US Publication No. 2005/0100893, US Publication No. 2004/0018491, US Publication No. 2003/0215821 and US Publication No. 2003/0207295.

[00159] In step **516**, bins with rare alleles (e.g., fetal alleles) are identified. Using the example described above, rare allele identification can be accomplished by first using the 22bp tags to sort the 20,000 genotypes into 500 bins which correspond to the individual wells of the original microtiter plates. Then, one can identify bins containing non-maternal alleles which correspond to wells that contained fetal cells. Determining the number bins with non-maternal alleles relative to the total number of its provides an accurate estimate of the number of fnRBCs that were present in the original enriched cell population. When a fetal cell is identified in a given bin, the non-maternal alleles can be detected by 40 independent SNPS s which provide an extremely high level of confidence in the result.

[00160] In step **518**, a condition such as trisomy is determined based on the rare cell polymorphism. For example, after identifying the ~10 bins that contain fetal cells, one can determine the ploidy of chromosomes 13, 18, 21 and X of such cells by comparing the ratio of maternal to paternal alleles for each of ~10 SNPs on each chromosome (X, 13, 18, 21). The ratios for the multiple SNPs on each chromosome can be combined (averaged) to increase the confidence of the aneuploidy call for that chromosome. In addition, the information from the ~10 independent bins containing fetal cells can also be combined to further increase the confidence of the call.

[00161] As described above, an enriched maternal sample with 500 cells can be split into 500 discrete locations such that each location contains one cell. If ten SNPs are analyzed in each of four different chromosomes, forty tagged MIP probes are added per discrete location to analyze forty different SNPs per cell. The forty SNPs are then amplified in each location using the primer element in the MIP probe as described above. All the amplicons from all the discrete locations are then pooled and analyzed using quantitative genotyping as describe above. In this example a total of 20,000 probes in a microarray are required to genotype the same 40 SNPs in each of the 500 discrete locations (4 chromosomes x 10 SNPs x 500 discrete locations).

[00162] The above embodiment can also be modified to provide for genotyping by hybridizing the nucleic acid tags to bead arrays as are commercially available by Illumina, Inc. and as described in US Patent Nos. 7,040,959; 7,035,740; 7033,754; 7,025,935, 6,998,274; 6, 942,968; 6,913,884; 6,890,764; 6,890,741; 6,858,394; 6,846,460; 6,812,005; 6,770,441; 6,663,832; 6,620,584; 6,544,732; 6,429,027; 6,396,995; 6,355,431 and US Publication Application Nos. 20060019258; 20050266432; 20050244870; 20050216207; 20050181394; 20050164246; 20040224353; 20040185482; 20030198573; 20030175773; 20030003490; 20020187515; and 20020177141; as well as Shen, R., et al. Mutation Research 573 70-82 (2005).

[00163] An overview of the use of nucleic acid tags is described in **Figure 7C**. After enrichment and amplification as described above, target genomic DNA regions are activated in **step 702** such that they may bind paramagnetic particles. In **step 703** assay oligonucleotides, hybridization buffer, and paramagnetic particles are combined with the activated DNA and allowed to hybridize (hybridization step). In some cases, three oligonucleotides are added for each SNP to be detected. Two of the three oligos are specific for each of the two alleles at a SNP position and are referred to as Allele-Specific Oligos (ASOs). A third oligo hybridizes several bases downstream from the SNP site and is referred to as the Locus-Specific Oligo (LSO). All three oligos contain regions of genomic complementarity (C1, C2, and C3) and universal PCR primer sites (P1, P2 and P3). The LSO also contains a unique address sequence (Address) that targets a particular bead type. In some cases, up to 1,536 SNPs may be interrogated in this manner. During the primer hybridization process, the assay oligonucleotides hybridize to the genomic DNA sample bound to paramagnetic particles. Because hybridization occurs prior to any amplification steps, no amplification bias is introduced into the assay. The above primers can further be modified to serve the two roles

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of determining the identity of the different SNPs and to determining the identity of the bin from which the genotype was derived. In **step 704**, following the hybridization step, several wash steps are performed reducing noise by removing excess and mis-hybridized oligonucleotides. Extension of the appropriate ASO and ligation of the extended product to the LSO joins information about the genotype present at the SNP site to the address sequence on the LSO. In **step 705**, the joined, full-length products provide a template for performing PCR reactions using universal PCR primers P1, P2, and P3. Universal primers P1 and P2 are labeled with two different labels (e.g., Cy3 and Cy5). Other labels that can be used include, chromophores, fluorescent moieties, enzymes, antigens, heavy metal, magnetic probes, dyes, phosphorescent groups, radioactive materials, chemiluminescent moieties, scattering or fluorescent nanoparticles, Raman signal generating moieties, or electrochemical detection moieties. In **step 706**, the single-stranded, labeled DNAs are eluted and prepared for hybridization. In step **707**, the single-stranded, labeled DNAs are hybridized to their complement bead type through their unique address sequence. Hybridization of the GoldenGate Assay products onto the Array Matrix of Beadchip allows for separation of the assay products in solution, onto a solid surface for individual SNP genotype readout. In **step 708**, the array is washed and dried. In **step 709**, a reader such as the BeadArray Reader is used to analyze signals from the label. For example, when the labels are dye labels such as Cy3 and Cy5, the reader can analyze the fluorescence signal on the Sentrix Array Matrix or BeadChip. In **step 710**, a computer readable medium having a computer executable logic recorded on it can be used in a computer to perform receive data from one or more quantified DNA genomic regions to automate genotyping clusters and callings. Expression detection and analysis using microarrays is described in part in Valk, P. J. et al. New England Journal of Medicine 350(16), 1617-28, 2004; Modlich, O. et al. Clinical Cancer Research 10(10), 3410-21, 2004; Onken, Michael D. et al. Cancer Res. 64(20), 7205-7209, 2004; Gardian, et al. J. Biol. Chem. 280(1), 556-563, 2005; Becker, M. et al. Mol. Cancer Ther. 4(1), 151-170, 2005; and Flechner, SM et al. Am J Transplant 4(9), 1475-89, 2004; as well as in US Patent Nos. 5,445,934; 5,700,637; 5,744,305; 5,945,334; 6,054,270; 6,140,044; 6,261,776; 6,291,183; 6,346,413; 6,399,365; 6,420,169; 6,551,817; 6,610,482; 6,733,977; and EP 619 321; 323 203.

[00164] In any of the embodiments herein, preferably, more than 1000, 5,000, 10,000, 50,000, 100,000, 500,000, or 1,000,000 SNPs are interrogated in parallel.

[00165] In another aspect of the invention, illustrated in part by **Figure 6**, the systems and methods herein can be used to diagnose, prognose, and monitor neoplastic conditions such as cancer in a patient. Examples of neoplastic conditions contemplated herein include acute lymphoblastic leukemia, acute or chronic lymphocytic or granulocytic tumor, acute myeloid leukemia, acute promyelocytic leukemia, adenocarcinoma, adenoma, adrenal cancer, basal cell carcinoma, bone cancer, brain cancer, breast cancer, bronchi cancer, cervical dysplasia, chronic myelogenous leukemia, colon cancer, epidermoid carcinoma, Ewing's sarcoma, gallbladder cancer, gallstone tumor, giant cell tumor, glioblastoma multiforma, hairy-cell tumor, head cancer, hyperplasia, hyperplastic comeal nerve tumor, in situ carcinoma, intestinal ganglioneuroma, islet cell tumor, Kaposi's sarcoma, kidney cancer, larynx cancer, leiomyomater tumor, liver cancer, lung cancer, lymphomas, malignant carcinoid, malignant hypercalcemia, malignant melanomas, marfanoid habitus tumor, medullary carcinoma, metastatic skin carcinoma, mucosal neuromas, mycosis fungoide, myelodysplastic syndrome, myeloma, neck cancer, neural tissue cancer, neuroblastoma, osteogenic sarcoma, osteosarcoma, ovarian tumor, pancreas cancer, parathyroid cancer, pheochromocytoma, polycythemia vera, primary brain tumor, prostate cancer, rectum cancer, renal cell tumor, retinoblastoma, rhabdomyosarcoma, seminoma, skin cancer, small-cell lung tumor, soft tissue sarcoma, squamous cell carcinoma, stomach cancer, thyroid cancer, topical skin lesion, veticulum cell sarcoma, and Wilm's tumor.

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[00166] Cancers such as breast, colon, liver, ovary, prostate, and lung as well as other tumors exfoliate epithelial cells into the bloodstream. The presence of an increased number epithelial cells is associated with an active tumor or other neoplastic condition, tumor progression and spread, poor response to therapy, relapse of disease, and/or decreased survival over a period of several years. Therefore, enumerating and/or analyzing epithelial cells and CTC's in the bloodstream can be used to diagnose, prognose, and/or monitor neoplastic conditions.

[00167] In step **600**, a sample is obtained from an animal such as a human. The human can be suspected of having cancer or cancer recurrence or may have cancer and is in need of therapy selection. The sample obtained is a mixed sample comprising normal cells as well as one or more CTCs, epithelial cells, endothelial cells, stem cells, or other cells indicative of cancer. In some cases, the sample is a blood sample. In some cases multiple samples are obtained from the animal at different points in time (e.g., regular intervals such as daily, or every 2, 3 or 4 days, weekly, bimonthly, monthly, bi-yearly or yearly.

[00168] In step **602**, the mixed sample is then enriched for epithelial cells or CTC's or other cell indicative of cancer. Epithelial cells that are exfoliated from solid tumors have been found in very low concentrations in the circulation of patients with advanced cancers of the breast, colon, liver, ovary, prostate, and lung, and the presence or relative number of these cells in blood has been correlated with overall prognosis and response to therapy. These epithelial cells which are in fact CTCs can be used as an early indicator of tumor expansion or metastasis before the appearance of clinical symptoms.

[00169] CTCs are generally larger than most blood cells. Therefore, one useful approach for obtaining CTCs in blood is to enrich them based on size, resulting in a cell population enriched in CTCs. Another way to enrich CTCs is by affinity separation, using antibodies specific for particular cell surface markers may be used. Useful endothelial cell surface markers include CD105, CD106, CD144, and CD146; useful tumor endothelial cell surface markers include TEM1, TEM5, and TEM8 (see, e.g., Carson-Walter et al., Cancer Res. 61:6649-6655 (2001)); and useful mesenchymal cell surface markers include CD133. Antibodies to these or other markers may be obtained from, e.g., Chemicon, Abcam, and R&D Systems.

[00170] In one example, a size-based separation module that enriches CTC's from a fluid sample (e.g., blood) comprises an array of obstacles that selectively deflect particles having a hydrodynamic size larger than 10 μm into a first outlet and particles having a hydrodynamic size smaller than 10 μm into a second outlet is used to enrich epithelial cells and CTC's from the sample.

[00171] In step **603**, the enriched product is split into a plurality of discrete sites, such as mirowells. Examplary microwells that can be used in the present invention include microplates having 1536 wells as well as those of lesser density (e.g., 96 and 384 wells). Microwell plate design contemplated herein include those have 14 outputs that can be automatically dispensed at the same time, as well as those with 16, 24, or 32 outputs such that e.g., 32 outputs can be dispenses simultaneously. **Figure 9** illustrates one embodiments of a microwell plate contemplated herein.

[00172] Dispensing of the cells into the various discrete sites is preferably automated. In some cases, about 1, 5, 10, or 15 μL of enriched sample is dispensed into each well. Preferably, the size of the well and volume dispensed into each well is such that only 1 cell is dispensed per well and only 1-5 or less than 3 cells can fit in each well.

[00173] An exemplary array for sample splitting is illustrated in **Figure 8A. Figure 8B** illustrates an isometric view and **Figure 8B** illustrates a top view and cross sectional view of such an array. A square array of wells is arranged such that each subsequent row or column of wells is identical to the previous row or column of wells, respectively. In some embodiments, an array of wells is configured in a substrate or plate that about 2.0 cm^2, 2.5 cm^2, 3 cm^2 or larger. The wells can be of any shape, e.g., round, square, or oval. The height or width of each well can be between 5-50 μm, 10-40 μm, or about 25 μm. The depth of each well can be up to 100, 80, 60, or 40 μm; and the radius

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between the centers of two wells in one column is between 10-60 μm, 20-50 μm, or about 35 μm. Using these configurations, an array of wells of area 2.5 cm^2 can have a at least 0.1 x 10^6 wells, 0.2 x 10^6 wells, 0.3 x 10^6 wells, 0.4 x 10^6 wells, or 0.5 x 10^6 wells.

[00174] In some embodiments, such as those illustrated in **Figure 8C** each well may have an opening at the bottom. The bottom opening is preferably smaller in size than the cells of interest. In this case, if the average radius of a CTC is about 10 μm, the bottom opening of each well can have a radius of up to 8, 7, 6, 5, 4, 3, 2 or 1 μm. The bottom opening allows for cells non-of interest and other components smaller than the cell of interest to be removed from the well using flow pressure, leaving the cells of interest behind in the well for further processing. Methods and systems for actuating removal of cells from discrete predetermined sites are disclosed in US Patent No. 6,692,952 and US Application Serial No. 11/146,581.

[00175] In some cases, the array of wells can be a micro-electro-mechanical system (MEMS) such that it integrates mechanical elements, sensors, actuators, and electronics on a common silicon substrate through microfabrication technology. Any electronics in the system can be fabricated using integrated circuit (IC) process sequences (e.g., CMOS, Bipolar, or BICMOS processes), while the micromechanical components are fabricated using compatible micromachining processes that selectively etch away parts of the silicon wafer or add new structural layers to form the mechanical and electromechanical devices. One example of a MEMS array of wells includes a MEMS isolation element within each well. The MEMS isolation element can create a flow using pressure and/or vacuum to increase pressure on cells and particles not of interest to escape the well through the well opening. In any of the embodiments herein, the array of wells can be coupled to a microscope slide or other substrate that allows for convenient and rapid optical scanning of all chambers (i.e. discrete sites) under a microscope. In some embodiments, a 1536-well microtiter plate is used for enhanced convenience of reagent addition and other manipulations.

[00176] In some cases, the enriched product can be split into wells such that each well is loaded with a plurality of leukocytes (e.g., more than 100, 200, 500, 1000, 2000, or 5000). In some cases, about 2500 leukocytes are dispensed per well, while random wells will have a single epithelial CTC or up to 2, 3, 4, or 5 epithelial cells or CTC's. Preferably, the probability of getting a single epithelial cell or CTC into a well is calculated such that no more than 1 CTC is loaded per well. The probability of dispensing CTC's from a sample into wells can be calculated using Poisson statistics. When dispensing a 15 mL sample into 1536 wellplate at 10 μL per well, it is not until the number of CTC's in the sample is > 100 that there is more than negligible probability of two or more CTC's being loaded into the sample well. **Figure 9** illustrates the probability density function of loading two CTC's into the same plate.

[00177] In step **604**, rare cells (e.g. epithelial cells or CTC's) or rare DNA is detected and/or analyzed in each well.

[00178] In some embodiments, detection and analysis includes enumerating epithelial cells and/or CTC's. CTCs typically have a short half-life of approximately one day, and their presence generally indicates a recent influx from a proliferating tumor. Therefore, CTCs represent a dynamic process that may reflect the current clinical status of patient disease and therapeutic response. Thus, in some embodiments, step **604** involves enumerating CTC and/or epithelial cells in a sample (array of wells) and determining based on their number if a patient has cancer, severity of condition, therapy to be used, or effectiveness of therapy administered.

[00179] In some cases, the method herein involve making a series of measurements, optionally made at regular intervals such as one day, two days, three days, one week, two weeks, one month, two months, three months, six months, or one year, one may track the level of epithelial cells present in a patient's bloodstream as a function of time. In the case of existing cancer patients, this provides a useful indication of the progression of the disease and assists medical practitioners in making appropriate therapeutic choices based on the increase, decrease, or lack of

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change in epithelial cells, e.g., CTCs, in the patient's bloodstream. For those at risk of cancer, a sudden increase in the number of cells detected may provide an early warning that the patient has developed a tumor. This early diagnosis, coupled with subsequent therapeutic intervention, is likely to result in an improved patient outcome in comparison to an absence of diagnostic information.

[00180] In some cases, more than one type of cell (e.g., epithelial, endothelial, etc.) can be enumerated and a determination of a ratio of numbers of cells or profile of various cells can be obtained to generate the diagnosis or prognosis.

[00181] Alternatively, detection of rare cells or rare DNA (e.g. epithelial cells or CTC's) can be made by detecting one or more cancer biomarkers, e.g., any of those listed in **Figure 10** in one or more cells in the array. Detection of cancer biomarkers can be accomplished using, e.g., an antibody specific to the marker or by detecting a nucleic acid encoding a cancer biomarker, e.g., listed in Figure 9.

[00182] In some cases single cell analysis techniques are used to analyze individual cells in each well. For example, single cell PCR may be performed on a single cell in a discrete location to detect one or more mutant alleles in the cell (Thornhill AR, J. Mol. Diag; (4) 11-29 (2002)) or a mutation in a gene listed in Figure 9. In-cell PCR, gene expression analysis can be performed even when the number of cells per well is very low (e.g. 1 cell per well) using techniques known in the art. (Giordano et al., Am. J. Pathol. 159:1231-1238 (2001), and Buckhaults et al., Cancer Res. 63:4144-4149 (2003). In some cases, single cell expression analysis can be performed to detection expression of one or more genes of interest (Liss B., Nucleic Acids Res., 30 (2002)) including those listed in Figure 9. Furthermore, ultra-deep sequencing can be performed on single cells using methods such as those described in Marguiles M., et al. Nature, "Genome sequencing in microfabricated high-density picolitre reactors." DOI 10.1038, in which whole genomes are fragmented, fragments are captured using common adapters on their own beads and within droplets of an emulsion, clonally amplified. Such ultra-deep sequending can also be used to detect mutations in genes associated with cancer, such as those listed in Figure 9. In addition, fluorescence in-situ hybridization can be used, e.g., to determine the tissue or tissues of origin of the cells being analyzed.

[00183] In some cases, morphological analyses are performed on the cells in each well. Morphological analyses include identification, quantification and characterization of mitochondrial DNA, telomerase, or nuclear matrix proteins. Parrella et al., Cancer Res. 61:7623-7626 (2001); Jones et al., Cancer Res. 61:1299-1304 (2001); Fliss et al., Science 287:2017-2019 (2000); and Soria et al., Clin. Cancer Res. 5:971-975 (1999). In particular, in some cases, the molecular analyses involves determining whether any mitochrondial abnormalities or whether perinuclear compartments are present. Carew et al., Mol. Cancer 1:9 (2002); and Wallace, Science 283:1482-1488 (1999).

[00184] A variety of cellular characteristics may be measured using any technique known in the art, including: protein phosphorylation, protein glycosylation, DNA methylation (Das et al., J. Clin. Oncol. 22:4632-4642 (2004)), microRNA levels (He et al., Nature 435:828-833 (2005), Lu et al., Nature 435:834-838 (2005), O'Donnell et al., Nature 435:839-843 (2005), and Calin et al., N. Engl. J. Med. 353:1793-1801 (2005)), cell morphology or other structural characteristics, e.g., pleomorphisms, adhesion, migration, binding, division, level of gene expression, and presence of a somatic mutation. This analysis may be performed on any number of cells, including a single cell of interest, e.g., a cancer cell.

[00185] In one embodiment, the cell(s) (such as fetal, maternal, epithelial or CTCs) in each well are lysed and RNA is extracted using any means known in the art. For example, The Quiagen RNeasy™ 96 bioRobot™ 8000 system can be used to automate high-throughput isolation of total RNA from each discrete site. Once the RNA is extracted reverse transcriptase reactions can be performed to generate cDNA sequences, which can then be used for performing multiplex PCR reactions on target genes. For example, 1 or more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or

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20 target genes can be amplified in the same reaction. When more than one target genes are used in the same amplification reaction, primers are chosen to be multiplexable (fairly uniform melting temperature, absence of cross-priming on the human genome, and absence of primer-primer interaction based on sequence analysis) with other pairs of primers. Multiple dyes and multi-color fluorescence readout may be used to increase the multiplexing capacity. Examples of dyes that can be used to label primers for amplification include, but are not limited to, chromophores, fluorescent moieties, enzymes, antigens, heavy metal, magnetic probes, dyes, phosphorescent groups, radioactive materials, chemiluminescent moeities, scattering or fluorescent nanoparticles, Raman signal generating moieties, and electrochemical detection moieties.

[00186] In another embodiment, fetal or maternal cells or nuclei are enriched using one or more methods disclosed herein. Preferably, fetal cells are enriched by flowing the sample through an array of obstacles that selectively directs particles or cells of different hydrodynamic sizes into different outlets such that fetal cells and cells larger than fetal cells are directed into a first outlet and one or more cells or particles smaller than the rare cells are directed into a second outlet.

[00187] Total RNA or poly-A mRNA is then obtained from enriched cell(s) (fetal or maternal cells) using purification techniques known in the art. Generally, about 1 μg – 2 μg of total RNA is sufficient. Next, a first-strand complementary DNA (cDNA) is synthesized using reverse transcriptase and a single T7-oligo(dT) primer. Next, a second-strand cDNA is synthesized using DNA ligase, DNA polymerase, and RNase enzyme. Next, the double stranded cDNA (ds-cDNA) is purified.

[00188] In another embodiment , total RNA is extracted from enriched cells (fetal cells or maternal cells). Next a, two one-quarter scale Message Amp II reactions (Ambion, Austin, Texas) are performed for each RNA extraction using 200 ng of total RNA. MessageAmp is a procedure based on antisense RNA (aRNA) amplification, and involves a series of enzymatic reactions resulting in linear amplification of exceedingly small amounts of RNA for use in array analysis. Unlike exponential RNA amplification methods, such as NASBA and RT-PCR, aRNA amplification maintains representation of the starting mRNA population. The procedure begins with total or poly(A) RNA that is reverse transcribed using a primer containing both oligo(dT) and a T7 RNA polymerase promoter sequence. After first-strand synthesis, the reaction is treated with RNase H to cleave the mRNA into small fragments. These small RNA fragments serve as primers during a second-strand synthesis reaction that produces a double-stranded cDNA template.

[00189] In some embodiments, cDNAs, which are reverse transcribed from mRNAs obtained from fetal or maternal cells, are tagged and sequenced. The type and abundance of the cDNAs can be used to determine whether a cell is a fetal cell (such as by the presence of Y chromosome specific transcripts) or whether the fetal cell has a genetic abnormality (such as aneuploidy, abundance or type of alternative transcripts or problems with DNA methylation or imprinting).

[00190] In one embodiment, PCR amplification can be performed on genes that are expressed in epithelial cells and not in normal cells, e.g., white blood cells or other cells remaining in an enriched product. Exemplary genes that can be analyzed according to the methods herein include EGFR, EpCAM, GA733-2, MUC-1, HER-2, Claudin-7 and any other gene identified in **Figure 10**.

[00191] For example, analysis of the expression level or pattern of such a polypeptide or nucleic acid, e.g., cell surface markers, genomic DNA, mRNA, or microRNA, may result in a diagnosis or prognosis of cancer.

[00192] In some embodiments, cDNAs, which are reverse transcribed from mRNAs obtained from fetal or maternal cells, are tagged and sequenced. The type and abundance of the cDNAs can be used to determine whether a cell is a

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fetal cell (such as by the presence of Y chromsome specific transcripts) or whether the fetal cell has a genetic abnormality (such as anueploidy, or problems with DNA methylation or imprinting).

[00193] In some embodiments, analysis step **604** involves identifying cells from a mixed sample that express genes which are not expressed in the non-rare cells (e.g. EGFR or EpCAM). For example, an important indicator for circulating tumor cells is the presence/expression of EGFR or EGF at high levels wherein non-cancerous epithelial cells will express EGFR or EGF at smaller amounts if at all.

[00194] In addition, for lung cancer and other cancers, the presence or absence of certain mutations in EGFR can be associated with diagnosis and/or prognosis of the cancer as well and can also be used to select a more effective treatment (see, e.g., International Publication WO 2005/094357). For example, many non-small cell lung tumors with EGFR mutations respond to small molecule EGFR inhibitors, such as gefitinib (Iressa; AstraZeneca), but often eventually acquire secondary mutations that make them drug resistant. In some embodiments, one can determine a therapy treatment for a patient by enriching epithelial cells and/or CTC's using the methods herein, splitting sample of cells (preferably so no more than 1 CTC is in a discrete location), and detecting one or more mutations in the EGFR gene of such cells. Exemplary mutations that can be analyzed include those clustered around the ATP-binding pocket of the EGFR TK domain, which are known to make cells susceptible to gefitinib inhibition. Thus, presence of such mutations supports a diagnosis of cancer that is likely to respond to treatment using gefitinib.

[00195] Many patients who respond to gefitinib eventually develop a second mutation, often a methionine-to-threonine substitution at position 790 in exon 20 of the TK domain. This type of mutation renders such patients resistant to gefitinib. Therefore, the present invention contemplates testing for this mutation as well to provide further diagnostic information.

[00196] Since many EGFR mutations, including all EGFR mutations in NSC lung cancer reported to date that are known to confer sensitivity or resistance to gefitinib, lie within the coding regions of exons 18 to 21, this region of the EGFR gene may be emphasized in the development of assays for the presence of mutations. Examples of primers that can be used to detect mutations in EGFR include those listed in **Figure 11.**

[00197] In step **605**, a determination is made as to the condition of a patient based on analysis made above. In some cases the patient can be diagnosed with cancer or lack thereof. In some cases, the patient can be prognosed with a particular type of cancer. In cases where the patient has cancer, therapy may be determined based on the types of mutations detected.

[00198] In another embodiment, cancer cells may be detected in a mixed sample (e.g. circulating tumor cells and circulating normal cells) using one or more of the sequencing methods described herein. Briefly, RNA is extracted from cells in each location and converted to cDNA as described above. Target genes are then amplified and high throughput ultra deep sequencing is performed to detect a mutation expression level associated with cancer.

VI. Computer Executable Logic

[00199] Any of the steps herein can be performed using computer program product that comprises a computer executable logic recorded on a computer readable medium. For example, the computer program can use data from target genomic DNA regions to determine the presence or absence of fetal cells in a sample and to determine fetal abnormalit(ies) in cells detected. In some embodiments, computer executable logic uses data input on STR or SNP intensities to determine the presence of fetal cells in a test sample and determine fetal abnormalities and/or conditions in said cells.

[00200] The computer program may be specially designed and configured to support and execute some or all of the functions for determining the presence of rare cells such as fetal cells or epithelial/CTC's in a mixed sample and abnormalities and/or conditions associated with such rare cells or their DNA including the acts of (i) controlling the

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splitting or sorting of cells or DNA into discrete locations (ii) amplifying one or more regions of genomic DNA e.g. trisomic region(s) and non-trisomic region(s) (particularly DNA polymorphisms such as STR and SNP) in cells from a mixed sample and optionally control sample, (iii) receiving data from the one or more genomic DNA regions analyzed (e.g. sequencing or genotyping data); (iv) identifying bins with rare (e.g. non-maternal) alleles, (v) identifying bins with rare (e.g. non-maternal) alleles as bins containing fetal cells or epithelial cells, (vi) determining number of rare cells (e.g. fetal cells or epithelial cells) in the mixed sample, (vii) detecting the levels of maternal and non-maternal alleles in identified fetal cells, (viii) detecting a fetal abnormality or condition in said fetal cells and/or (ix) detecting a neoplastic condition and information concerning such condition such as its prevalence, origin, susceptibility to drug treatment(s), etc. In particular, the program can fit data of the quantity of allele abundance for each polymorphism into one or more data models. One example of a data model provides for a determination of the presence or absence of aneuploidy using data of amplified polymorphisms present at loci in DNA from samples that are highly enriched for fetal cells. The determination of presence of fetal cells in the mixed sample and fetal abnormalities and/or conditions in said cells can be made by the computer program or by a user.

[00201] In one example, let 'f' be the fetal/maternal DNA copy ratio in a particular PCR reaction. Trisomy increases the ratio of maternal to paternal alleles by a factor 1+f/2. PCR efficiencies vary from allele to allele within a locus by a mean square error in the logarithm given by σ_{allele}^2, and vary from locus to locus by σ_{locus}^2, where this second variance is apt to be larger due to differences in primer efficiency. N_a is the loci per suspected aneuploid chromosome and N_c is the control loci. If the mean of the two maternal allele strengths at any locus is 'm' and the paternal allele strength is 'p,' then the squared error expected is the mean of the ln(ratio(m/p)), where this mean is taken over N loci is given by $2(\sigma_{allele}^2)/N$. When taking the difference of this mean of ln(ratio(m/p)) between a suspected aneuploidy region and a control region, the error in the difference is given by

$$\sigma_{diff}^2 = 2(\sigma_{allele}^2)/N_a + 2(\sigma_{allele}^2)/N_c \quad (1)$$

[00202] For a robust detection of aneuploidy we require

$$3\sigma_{diff} < f/2.$$

[00203] For simplicity, assuming $N_a = N_c = N$ in Equation 1, this gives the requirement

$$6\sigma_{allele}/N^{1/2} < f/2, \quad (3)$$

or a minimum N of

$$N = 144(\sigma_{allele}/f)^2 \quad (4)$$

[00204] In the context of trisomy detection, the suspected aneuploidy region is usually the entire chromosome and N denotes the number of loci per chromosome. For reference, Equation 3 is evaluated for N in **Table 2** for various values of σ_{allele} and f.

[00205] The role of the sequencing is to measure the allele abundances output from the amplification step. It is desirable to do this without adding significantly more error due to the Poisson statistics of selecting only a finite number of amplicons for sequencing. The rms error in the ln(abundance) due to Poisson statistics is approximately $(N_{reads})^{-1/2}$. It is desirable to keep this value less than or equal to the PCR error σ_{allele}. Thus, a typical paternal allele needs to be allocated at least $(\sigma_{allele})^{-2}$ reads. The maternal alleles, being more abundant, do not add appreciably to this error when forming the ratio estimate for m/p. The mixture input to sequencing contains amplicons from N_{loci} loci of which roughly an abundance fraction f/2 are paternal alleles. Thus, the total required number of reads for each of the aliquot wells is given approximately by $2N_{loci}/(f\,\sigma_{allele}^2)$. Combining this result with Equation 4, it is found a total number of reads over all the wells given approximately by

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$N_{reads} = 288\ N_{wells}\ f^3$. Thus, the program can determine the total number of reads that need to be obtained for determining the presence or absence of aneuploidy in a patient sample.

[00206] The computer program can work in any computer that may be any of a variety of types of general-purpose computers such as a personal computer, network server, workstation, or other computer platform now or later developed. In some embodiments, a computer program product is described comprising a computer usable medium having the computer executable logic (computer software program, including program code) stored therein. The computer executable logic can be executed by a processor, causing the processor to perform functions described herein. In other embodiments, some functions are implemented primarily in hardware using, for example, a hardware state machine. Implementation of the hardware state machine so as to perform the functions described herein will be apparent to those skilled in the relevant arts.

[00207] In one embodiment, the computer executing the computer logic of the invention may also include a digital input device such as a scanner. The digital input device can provide an image of the target genomic DNA regions (e.g. DNA polymorphism, preferably STRs or SNPs) according to method of the invention. For instance, the scanner can provide an image by detecting fluorescent, radioactive, or other emissions; by detecting transmitted, reflected, or scattered radiation; by detecting electromagnetic properties or characteristics; or by other techniques. Various detection schemes are employed depending on the type of emissions and other factors. The data typically are stored in a memory device, such as the system memory described above, in the form of a data file.

[00208] In one embodiment, the scanner may identify one or more labeled targets. For instance, in the genotyping analysis described herein a first DNA polymorphism may be labeled with a first dye that fluoresces at a particular characteristic frequency, or narrow band of frequencies, in response to an excitation source of a particular frequency. A second DNA polymorphisms may be labeled with a second dye that fluoresces at a different characteristic frequency. The excitation sources for the second dye may, but need not, have a different excitation frequency than the source that excites the first dye, e.g., the excitation sources could be the same, or different, lasers.

[00209] In one embodiment, a human being may inspect a printed or displayed image constructed from the data in an image file and may identify the data (e.g. fluorescence from microarray) that are suitable for analysis according to the method of the invention. In another embodiment, the information is provided in an automated, quantifiable, and repeatable way that is compatible with various image processing and/or analysis techniques.

[00210] Another aspect of the invention is kits which permit the enrichment and analysis of the rare cells present in small qualities in the samples. Such kits may include any materials or combination of materials described for the individual steps or the combination of steps ranging from the enrichment through the genetic analysis of the genomic material. Thus, the kits may include the arrays used for size-based separation or enrichment, labels for uniquely labeling each cell, the devices utilized for splitting the cells into individual addressable locations and the reagents for the genetic analysis. For example, a kit might contain the arrays for size-based separation, unique labels for the cells and reagents for detecting polymorphisms including STRs or SNPs, such as reagents for performing PCR.

[00211] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

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EXAMPLES

Example 1. Separation of Fetal Cord Blood

[00212] Figure 1E shows a schematic of the device used to separate nucleated cells from fetal cord blood.

[00213] *Dimensions:* 100 mm × 28 mm × 1mm

[00214] *Array design*: 3 stages, gap size = 18, 12 and 8 μm for the first, second and third stage, respectively.

[00215] *Device fabrication*: The arrays and channels were fabricated in silicon using standard photolithography and deep silicon reactive etching techniques. The etch depth is 140 μm. Through holes for fluid access are made using KOH wet etching. The silicon substrate was sealed on the etched face to form enclosed fluidic channels using a blood compatible pressure sensitive adhesive (9795, 3M, St Paul, MN).

[00216] *Device packaging*: The device was mechanically mated to a plastic manifold with external fluidic reservoirs to deliver blood and buffer to the device and extract the generated fractions.

[00217] *Device operation*: An external pressure source was used to apply a pressure of 2.0 PSI to the buffer and blood reservoirs to modulate fluidic delivery and extraction from the packaged device.

[00218] *Experimental conditions*: Human fetal cord blood was drawn into phosphate buffered saline containing Acid Citrate Dextrose anticoagulants. 1mL of blood was processed at 3 mL/hr using the device described above at room temperature and within 48 hrs of draw. Nucleated cells from the blood were separated from enucleated cells (red blood cells and platelets), and plasma delivered into a buffer stream of calcium and magnesium-free Dulbecco's Phosphate Buffered Saline (14190-144, Invitrogen, Carlsbad, CA) containing 1% Bovine Serum Albumin (BSA) (A8412-100ML, Sigma-Aldrich, St Louis, MO) and 2 mM EDTA (15575-020, Invitrogen, Carlsbad, CA).

[00219] *Measurement techniques*: Cell smears of the product and waste fractions (Figure 12A-12B) were prepared and stained with modified Wright-Giemsa (WG16, Sigma Aldrich, St. Louis, MO).

[00220] *Performance*: Fetal nucleated red blood cells were observed in the product fraction (Figure 12A) and absent from the waste fraction (Figure 12B).

Example 2. Isolation of Fetal Cells from Maternal blood

[00221] The device and process described in detail in Example 1 were used in combination with immunomagnetic affinity enrichment techniques to demonstrate the feasibility of isolating fetal cells from maternal blood.

[00222] *Experimental conditions*: blood from consenting maternal donors carrying male fetuses was collected into K_2EDTA vacutainers (366643, Becton Dickinson, Franklin Lakes, NJ) immediately following elective termination of pregnancy. The undiluted blood was processed using the device described in Example 1 at room temperature and within 9 hrs of draw. Nucleated cells from the blood were separated from enucleated cells (red blood cells and platelets), and plasma delivered into a buffer stream of calcium and magnesium-free Dulbecco's Phosphate Buffered Saline (14190-144, Invitrogen, Carlsbad, CA) containing 1% Bovine Serum Albumin (BSA) (A8412-100ML, Sigma-Aldrich, St Louis, MO). Subsequently, the nucleated cell fraction was labeled with anti-CD71 microbeads (130-046-201, Miltenyi Biotech Inc., Auburn, CA) and enriched using the MiniMACS™ MS column (130-042-201, Miltenyi Biotech Inc., Auburn, CA) according to the manufacturer's specifications. Finally, the CD71-positive fraction was spotted onto glass slides.

[00223] *Measurement techniques*: Spotted slides were stained using fluorescence *in situ* hybridization (FISH) techniques according to the manufacturer's specifications using Vysis probes (Abbott Laboratories, Downer's Grove, IL). Samples were stained from the presence of X and Y chromosomes. In one case, a sample prepared from a known Trisomy 21 pregnancy was also stained for chromosome 21.

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[00224] *Performance*: Isolation of fetal cells was confirmed by the reliable presence of male cells in the CD71-positive population prepared from the nucleated cell fractions (Figures 13A–13F). In the single abnormal case tested, the trisomy 21 pathology was also identified (Figure 14).

Example 3. Confirmation of the Presence of Male Fetal Cells in Enriched Samples.

[00225] Confirmation of the presence of a male fetal cell in an enriched sample is performed using qPCR with primers specific for DYZ, a marker repeated in high copy number on the Y chromosome. After enrichment of fnRBC by any of the methods described herein, the resulting enriched fnRBC are binned by dividing the sample into 100 PCR wells. Prior to binning, enriched samples may be screened by FISH to determine the presence of any fnRBC containing an aneuploidy of interest. Because of the low number of fnRBC in maternal blood, only a portion of the wells will contain a single fnRBC (the other wells are expected to be negative for fnRBC). The cells are fixed in 2% Paraformaldehyde and stored at 4°C. Cells in each bin are pelleted and resuspended in 5 µl PBS plus 1 µl 20 mg/ml Proteinase K (Sigma #P-2308). Cells are lysed by incubation at 65°C for 60 minutes followed by inactivation of the Proteinase K by incubation for 15 minutes at 95°C. For each reaction, primer sets (DYZ forward primer TCGAGTGCATTCCATTCCG; DYZ reverse primer ATGGAATGGCATCAAACGGAA; and DYZ Taqman Probe 6FAM-TGGCTGTCCATTCCA-MGBNFQ), TaqMan Universal PCR master mix, No AmpErase and water are added. The samples are run and analysis is performed on an ABI 7300: 2 minutes at 50°C, 10 minutes 95°C followed by 40 cycles of 95°C (15 seconds) and 60°C (1 minute). Following confirmation of the presence of male fetal cells, further analysis of bins containing fnRBC is performed. Positive bins may be pooled prior to further analysis.

[00226] **Figure 30** shows the results expected from such an experiment. The data in **Figure 30** was collected by the following protocol. Nucleated red blood cells were enriched from cord cell blood of a male fetus by sucrose gradient two Heme Extractions (HE). The cells were fixed in 2% paraformaldehyde and stored at 4°C. Approximately 10 x 1000 cells were pelleted and resuspended each in 5 µl PBS plus 1 µl 20 mg/ml Proteinase K (Sigma #P-2308). Cells were lysed by incubation at 65°C for 60 minutes followed by a inactivation of the Proteinase K by 15 minute at 95°C. Cells were combined and serially diluted 10-fold in PBS for 100, 10 and 1 cell per 6 µl final concentration were obtained. Six µl of each dilution was assayed in quadruplicate in 96 well format. For each reaction, primer sets (0.9uM DYZ forward primer TCGAGTGCATTCCATTCCG; 0.9uM DYZ reverse primer ATGGAATGGCATCAAACGGAA; and 0.5uM DYZ TaqMan Probe 6FAM-TGGCTGTCCATTCCA-MGBNFQ), TaqMan Universal PCR master mix, No AmpErase and water were added to a final volume of 25 µl per reaction. Plates were run and analyzed on an ABI 7300: 2 minutes at 50°C, 10 minutes 95°C followed by 40 cycles of 95°C (15 seconds) and 60°C (1 minute). These results show that detection of a single fnRBC in a bin is possible using this method.

Example 4. Confirmation of the presence of fetal cells in enriched samples by STR analysis.

[00227] Maternal blood is processed through a size-based separation module, with or without subsequent MHEM enhancement of fnRBCs. The enhanced sample is then subjected to FISH analysis using probes specific to the aneuploidy of interest (e.g., trisomy 13, trisomy 18, and XYY). Individual positive cells are isolated by "plucking" individual positive cells from the enhanced sample using standard micromanipulation techniques. Using a nested PCR protocol, STR marker sets are amplified and analyzed to confirm that the FISH-positive aneuploid cell(s) are of fetal origin. For this analysis, comparison to the maternal genotype is typical. An example of a potential resulting data set is shown in **Table 3**. Non-maternal alleles may be proven to be paternal alleles by paternal genotyping or genotyping of known fetal tissue samples. As can be seen, the presence of paternal alleles in the resulting cells,

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demonstrates that the cell is of fetal origin (cells # 1, 2, 9, and 10). Positive cells may be pooled for further analysis to diagnose aneuploidy of the fetus, or may be further analyzed individually.

Table 3. STR locus alleles in maternal and fetal cells

DNA Source	STR locus D14S	STR locus D16S	STR locus D8S	STR locus F13B	STR locus vWA
Maternal alleles	14, 17	11, 12	12, 14	9, 9	16, 17
Cell #1 alleles		8			19
Cell #2 alleles	17		15		
Cell #3 alleles			14		
Cell #4 alleles					
Cell #5 alleles	17	12		9	
Cell #6 alleles					
Cell #7 alleles					19
Cell #8 alleles					
Cell #9 alleles	17		14	7, 9	17, 19
Cell #10 alleles			15		

Example 5. Confirmation of the presence of fetal cells in enriched samples by SNP analysis.

[00228] Maternal blood is processed through a size-based separation module, with or without subsequent MHEM enhancement of fnRBCs. The enhanced sample is then subjected to FISH analysis using probes specific to the aneuploidy of interest (e.g., triploidy 13, triploidy 18, and XYY). Samples testing positive with FISH analysis are then binned into 96 microtiter wells, each well containing 15 µl of the enhanced sample. Of the 96 wells, 5-10 are expected to contain a single fnRBC and each well should contain approximately 1000 nucleated maternal cells (both WBC and mnRBC). Cells are pelleted and resuspended in 5µl PBS plus 1µl 20 mg/ml Proteinase K (Sigma #P-2308). Cells are lysed by incubation at 65°C for 60 minutes followed by a inactivation of the Proteinase K by 15 minute at 95°C.

[00229] In this example, the maternal genotype (BB) and fetal genotype (AB) for a particular set of SNPs is known. The genotypes A and B encompass all three SNPs and differ from each other at all three SNPs. The following sequence from chromosome 7 contains these three SNPs (rs7795605, rs7795611 and rs7795233 indicated in brackets, respectively):

(ATGCAGCAAGGCACAGACTAA[G/A]CAAGGAGA[G/C]GCAAAATTTTC[A/G]TAGGGGAGAGAAATGG GTCATT).

[00230] In the first round of PCR, genomic DNA from binned enriched cells is amplified using primers specific to the outer portion of the fetal-specific allele A and which flank the interior SNP (forward primer ATGCAGCAAGGCACAGACTACG; reverse primer AGAGGGGAGAGAAATGGGTCATT). In the second round of PCR, amplification using real time SYBR Green PCR is performed with primers specific to the inner portion of allele A and which encompass the interior SNP (forward primer CAAGGCACAGACTAAGCAAGGAGAG; reverse primer GGCAAAATTTTCATAGGGGAGAGAAATGGGTCATT).

[00231] Expected results are shown in **Figure 31**. Here, six of the 96 wells test positive for allele A, confirming the presence of cells of fetal origin, because the maternal genotype (BB) is known and cannot be positive for allele A. DNA from positive wells may be pooled for further analysis or analyzed individually.

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Example 6. Quantitative Genotyping Using Molecular Inversion Probes for Trisomy Diagnosis on Fetal Cells

[00232] Fetal cells or nuclei can be isolated as described in the enrichment section or as described in example 1. Quantitative genotyping can then be used to detect chromosome copy number changes. Figure 5 depicts a flow chart depicting the major steps involved in detecting chromosome copy number changes using the methods described herein. For example, the enrichment process described in example 1 may generate a final mixture containing approximately 500 maternal white blood cells (WBCs), approximately 100 maternal nuclear red blood cells (mnBCs), and a minimum of approximately 10 fetal nucleated red blood cells (fnRBCs) starting from an initial 20 ml blood sample taken late in the first trimester. The output of the enrichment procedure would be divided into separate wells of a microtiter plate with the number of wells chosen so no more than one cell or genome copy is located per well, and where some wells may have no cell or genome copy at all.

[00233] *Perform multiplex PCR and nested PCR:* PCR primer pairs for multiple (40 – 100) highly polymorphic SNPs can then be added to each well in the microtiter plate. For example, SNPs primers can be designed along chromosomes 13, 18, 21 and X to detect the most frequent aneuploidies, and along control regions of the genome where aneuploidy is not expected. Multiple (~10) SNPs would be designed for each chromosome of interest to allow for non-informative genotypes and to ensure accurate results. The SNPs listed in the Table below can be used to performed analysis and associated PCR primers can be designed as described below.

SNPs that can be used for fetal cell analysis			
Chromosome 13	Chromosome 18	Chromosome 21	Chromosome X
refSNP rs9510053	refSNP rs584853	refSNP rs469000	refSNP rs6608727
refSNP rs7339372	refSNP rs2345588	refSNP rs7278903	refSNP rs2015487
refSNP rs9580269	refSNP rs9973072	refSNP rs1004044	refSNP rs5953330
refSNP rs724946	refSNP rs7504787	refSNP rs11910419	refSNP rs5953330
refSNP rs11842845	refSNP rs4303617	refSNP rs2832890	refSNP rs1984695
refSNP rs7490040	refSNP rs9947441	refSNP rs1785477	refSNP rs5906775
refSNP rs12430585	refSNP rs2912334	refSNP rs2250226	refSNP rs5951325
refSNP rs713280	refSNP rs11659665	refSNP rs2243594	refSNP rs11798710
refSNP rs202090	refSNP rs8098249	refSNP rs10483087	refSNP rs4898352
refSNP rs5000966	refSNP rs12968582	refSNP rs855262	refSNP rs5987079

[00234] PCR primers would be chosen to be multiplexible with other pairs (fairly uniform melting temperature, absence of cross-priming on the human genome, and absence of primer-primer interaction based on sequence analysis). The primers would be designed to generate amplicons 70 - 100 bp in size to increase the performance of the multiplex PCR. The primers would contain a 22 bp tag on the 5' which is used in the genotyping analysis. Multiplex PCR protocols can be performed as described in Findlay et al. Molecular Cell Endocrinology 183 (2001) S5-S12. Primer concentrations can vary from 0.7 pmoles to 60 pmoles per reaction. Briefly, PCRs are performed in a total volume of 25 µl per well, Taq polymerase buffer (Perkin-Elmer), 200 µM dNTPs, primer, 1.5 mM MgCl2 and 0.6 units AmpliTaq (Perkin-Elmer). After denaturation at 95 °C for 5 min, 41 cycles at 94, 60 and 72 °C for 45 s are performed in a MJ DNA engine thermal cycler. The amplification can be run with an annealing temperature different that 60 °C depending on the primer pair being amplified. Final extension can be for 10 min.

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[00235] A second of round of PCR using nested primers may be performed to ensure optimal performance of the multiplex amplification. Two ul aliquot of each PCR reaction is diluted 40 fold (to 80 ul total) with nuclease free water from the PCR kit. A no template or negative control is generated to test for contamination. The amplification with the nested PCR primers is run with an annealing temperature of 60° C - 68° C depending on the primer pair being amplified.

Nested PCR cycle		
Step	Temp (C)	Time (mins)
1.0	95	0.5
2.0	94	0.5
3.0	X	1.5
4.0	72	1.5
5.0	cycle to step 2, 44 times	
6.0	72	10

[00236] ...ology developed by ... typical MIP assay, each SNP would be assigned a 22bp DNA tag which allows the SNP to be uniquely identified during the highly parallel genotyping assay. In this example, the DNA tags serve two roles: (1) determine the identity of the different SNPs and (2) determine the identity of the well from which the genotype was derived. For example, a total of 20,000 tags would be required to genotype the same 40 SNPs in 500 wells different wells (4 chromosomes x 10 SNPs x 500 wells)

[00237] The tagged MIP probes would be combined with the amplicons from the initial multiplex single-cell PCR (or nested PCR) and the genotyping reactions would be performed. The probe/template mix would be divided into 4 tubes each containing a different nucleotide (e.g. G, A, T or C). Following an extension and ligation step, the mixture would be treated with exonuclease to remove all linear molecules and the tags of the surviving circular molecules would be amplified using PCR. The amplified tags form all of the bins would then be pooled and hybridized to a single DNA microarray containing the complementary sequences to each of the 20,000 tags.

[00238] *Identify bins with non-maternal alleles (e.g. fetal cells):* The first step in the data analysis procedure would be to use the 22bp tags to sort the 20,000 genotypes into bins which correspond to the individual wells of the original microtiter plates. The second step would be to identify bins contain non-maternal alleles which correspond to wells that contained fetal cells. Determining the number bins with non-maternal alleles relative to the total number of bins would provide an accurate estimate of the number of fnRBCs that were present in the original enriched cell population. When a fetal cell is identified in a given bin, the non-maternal alleles would be detected by 40 independent SNPs which provide an extremely high level of confidence in the result.

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[00239] *Detect ploidy for chromosomes 13, 18, and 21*: After identifying approximately 10 bins that contain fetal cells, the next step would be to determine the ploidy of chromosomes 13, 18, 21 and X by comparing ratio of maternal to paternal alleles for each of the 10 SNPs on each chromosome. The ratios for the multiple SNPs on each chromosome can be combined (averaged) to increase the confidence of the aneuploidy call for that chromosome. In addition, the information from the approximate 10 independent bins containing fetal cells can also be combined to further increase the confidence of the call.

Example 7. Ultra-deep Sequencing for Trisomy Diagnosis on Fetal Cells

[00240] Fetal cells or nuclei can be isolated as described in the enrichment section or as described in example 1. The enrichment process described in example 1 may generate a final mixture containing approximately 500 maternal white blood cells (WBCs), approximately 100 maternal nuclear red blood cells (mnBCs), and a minimum of approximately 10 fetal nucleated red blood cells (fnRBCs) starting from an initial 20 ml blood sample taken late in the first trimester. The output of the enrichment procedure would be divided into separate wells of a microtiter plate with the number of wells chosen so no more than one cell or genome copy is located per well, and where some wells may have no cell or genome copy at all.

[00241] *Perform multiplex PCR and Ultra-Deep Sequencing with bin specific tags:* PCR primer pairs for highly polymorphic STR loci (multiple loci per chromosome of interest) are then added to each well in the microtiter plate. The polymorphic STRs listed in the Table below can be used to performed analysis and associated PCR primers can be designed.

STR loci that can be used for fetal cell analysis	
MARKER	CHROMOSOME LOCATION
D21S1414	21q21
MBP	18q23-ter
D13S634	13q14.3-22
D13S631	13q31-32
D18S535	18q12.2-12.3
D21S1412	21(S171-S198)
D21S1411	21q22.3
D21S11	21q21
D18S386	18q22.1-18q22.2
D13S258	13q21.2-13q31
D13S303	13q22-13q31
D18S1002	18q11

[00242] The primers for each STR will have two important features. First, each of the primers will contain a common ~18bp sequence on the 5' end which is used for the subsequent DNA cloning and sequencing procedures. Second, each well in the microtiter plate is assigned a unique ~6bp DNA tag sequence which is incorporated into the middle part of the upstream primer for each of the different STRs. The DNA tags make it possible to pool all of the STR amplicons following the multiplex PCR which makes it possible to analyze the amplicons in parallel more cost effectively during the ultra-deep sequencing procedure. DNA tags of length ~ 6 bp provide a compromise between information content (4096 potential bins) and the cost of synthesizing primers.

[00243] Multiplex PCR protocols can be performed as described in Findlay et al. Molecular Cell Endocrinology 183 (2001) S5-S12. Primer concentrations can vary from 0.7 pmoles to 60 pmoles per reaction. Briefly, PCRs are performed in a total volume of 25 µl per well, Taq polymerase buffer (Perkin-Elmer), 200 µM dNTPs, primer, 1.5 mM MgCl2 and 0.6 units AmpliTaq (Perkin-Elmer). After denaturation at 95 °C for 5 min, 41 cycles at 94, 60 and

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72 °C for 45 s are performed in a MJ DNA engine thermal cycler. The amplification can be run with an annealing temperature different that 60 °C depending on the primer pair being amplified. Final extension can be for 10 min.

[00244] Following PCR, the amplicons from each of the wells in the microtiter plate are pooled, purified and analyzed using a single-molecule sequencing strategy as described in Margulies et al. Nature 437(2005) 376-380. Briefly, the amplicons are diluted and mixed with beads such that each bead captures a single molecule of the amplified material. The DNA-carrying beads are isolated in separate 100 um aqueous droplets made through the creation of a PCR-reation-mixture-in-oil emulsion. The DNA molecule on each bead is then amplified to generate millions of copies of the sequence, which all remain bound to the bead. Finally, the beads are placed into a highly parallel sequencing-by-synthesis machine which can generate over 400,000 sequence reads (~100bp per read) in a single 4 hour run.

[00245] Ultra-deep sequencing provides an accurate and quantitative way to measure the allele abundances for each of the STRs. The total required number of reads for each of the aliquot wells is determined by the number of STRs and the error rates of the multiplex PCR and the Poisson sampling statistics associated with the sequencing procedures. Statistical models which may account for variables in amplification can be used to detect ploidy changes with high levels of confidence. Using this statistical model it can be predicted that ~100,000 to 300,000 sequence reads will be required to analyze each patient, with ~3 to 10 STR loci per chromosome. Specifically, ~33 reads for each of 12 STRs in each of the individual wells of the microtiter plate will be read (33 reads x 12 STRs per well x 500 wells = 200,000 reads).

[00246] *Identify bins with non-maternal alleles (e.g. fetal cells)*: The first step in the data analysis procedure would be to use the 6bp DNA tags to sort the 200,000 sequence reads into bins which correspond to the individual wells of the microtiter plates. The ~400 sequence reads from each of the bins would then be separated into the different STR groups using standard sequence alignment algorithms. The aligned sequences from each of the bins would then be analyzed to identify non-maternal alleles. These can be identified in one of two ways. First, an independent blood sample fraction known to contain only maternal cells can be analyzed as described above. This sample can be a white blood cell fraction (which will contain only negligible numbers of fetal cells), or simply a dilution of the original sample before enrichment. Alternatively, the genotype profiles for all the wells can be similarity-clustered to identify the dominant pattern associated with maternal cells. In either approach, the detection of non-maternal alleles then determines which wells in the initial microtiter plate contained fetal cells. Determining the number bins with non-maternal alleles relative to the total number of bins provides an estimate of the number of fetal cells that were present in the original enriched cell population. Bins containing fetal cells would be identified with high levels of confidence because the non-maternal alleles are detected by multiple independent STRs.

[00247] *Detect ploidy for chromosomes 13, 18, and 21:* After identifying the bins that contained fetal cells, the next step would be to determine the ploidy of chromosomes 13, 18 and 21 by comparing the ratio of maternal to paternal alleles for each of the STRs. Again, for each bin there will be ~33 sequence reads for each of the 12 STRs. In a normal fetus, a given STR will have 1:1 ratio of the maternal to paternal alleles with approximately 16 sequence reads corresponding to each allele (normal diallelic). In a trisomic fetus, three doses of an STR marker can be detected either as three alleles with a 1:1:1 ratio (trisomic triallelic) or two alleles with a ratio of 2:1 (trisomic diallelic). In rare instances all three alleles may coincide and the locus will not be informative for that individual patient. The information from the different STRs on each chromosome can be combined to increase the confidence of a given aneuploidy call. In addition, the information from the independent bins containing fetal cells can also be combined to further increase the confidence of the call.

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Example 8. Sequencing for Trisomy Diagnosis on Fetal Cells

[00248] Fetal cells or nuclei can be isolated as described in the enrichment section or as described in example 1 and 2. Sequencing methods can then be used to detect chromosome copy number changes. **Figure 4** depicts a flow chart depicting the major steps involved in detecting chromosome copy number changes using the methods described herein. For example, the enrichment process described in example 1 may generate a final mixture containing approximately 500 maternal white blood cells (WBCs), approximately 100 maternal nuclear red blood cells (mnBCs), and a minimum of approximately 10 fetal nucleated red blood cells (fnRBCs) starting from an initial 20 ml blood sample taken late in the first trimester. The output of the enrichment procedure would be divided into separate wells of a microtiter plate with the number of wells chosen so no more than one cell or genome copy is located per well, and where some wells may have no cell or genome copy at all.

[00249] *Perform multiplex PCR and Sequencing with bin specific tags:*

[00250] PCR primer pairs for highly polymorphic STR loci (multiple loci per chromosome of interest) can be added to each well in the microtiter plate. For example, STRs could be designed along chromosomes 13, 18, 21 and X to detect the most frequent aneuploidies, and along control regions of the genome where aneuploidy is not expected. Typically, four or more STRs should be analyzed per chromosome of interest to ensure accurate detection of aneuploidy.

[00251] The primers for each STR can be designed with two important features. First, each primer can contain a common ~18bp sequence on the 5' end which can be used for the subsequent DNA cloning and sequencing procedures. Second, each well in the microtiter plate can be assigned a unique ~6bp DNA tag sequence which can be incorporated into the middle part of the upstream primer for each of the different STRs. The DNA tags make it possible to pool all of the STR amplicons following the multiplex PCR, which makes possible to analyze the amplicons in parallel during the ultra-deep sequencing procedure. Furthermore, nested PCR strategies for the STR amplification can achieve higher reliability of amplification from single cells.

[00252] Sequencing can be performed using the classic Sanger sequencing method or any other method known in the art.

[00253] For example, sequencing can occur by sequencing-by-synthesis, which involves inferring the sequence of the template by synthesizing a strand complementary to the target nucleic acid sequence. Sequence-by-synthesis can be initiated using sequencing primers complementary to the sequencing element on the nucleic acid tags. The method involves detecting the identity of each nucleotide immediately after (substantially real-time) or upon (real-time) the incorporation of a labeled nucleotide or nucleotide analog into a growing strand of a complementary nucleic acid sequence in a polymerase reaction. After the successful incorporation of a label nucleotide, a signal is measured and then nulled by methods known in the art. Examples of sequence-by-synthesis methods are described in U.S. Application Publication Nos. 2003/0044781, 2006/0024711, 2006/0024678 and 2005/0100932. Examples of labels that can be used to label nucleotide or nucleotide analogs for sequencing-by-synthesis include, but are not limited to, chromophores, fluorescent moieties, enzymes, antigens, heavy metal, magnetic probes, dyes, phosphorescent groups, radioactive materials, chemiluminescent moeities, scattering or fluorescent nanoparticles, Raman signal generating moieties, and electrochemical detection moieties. Sequencing-by-synthesis can generate at least 1,000, at least 5,000, at least 10,000, at least 20,000, 30,000, at least 40,000, at least 50,000, at least 100,000 or at least 500,000 reads per hour. Such reads can have at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 120 or at least 150 bases per read.

[00254] Another sequencing method involves hybridizing the amplified genomic region of interest to a primer complementary to it. This hybridization complex is incubated with a polymerase, ATP sulfurylase, luciferase,

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apyrase, and the substrates luciferin and adenosine 5' phosphosulfate. Next, deoxynucleotide triphosphates corresponding to the bases A, C, G, and T (U) are added sequentially. Each base incorporation is accompanied by release of pyrophosphate, converted to ATP by sulfurylase, which drives synthesis of oxyluciferin and the release of visible light. Since pyrophosphate release is equimolar with the number of incorporated bases, the light given off is proportional to the number of nucleotides adding in any one step. The process is repeated until the entire sequence is determined.

[00255] Yet another sequencing method involves a four-color sequencing by ligation scheme (degenerate ligation), which involves hybridizing an anchor primer to one of four positions. Then an enzymatic ligation reaction of the anchor primer to a population of degenerate nonamers that are labeled with fluorescent dyes is performed. At any given cycle, the population of nonamers that is used is structure such that the identity of one of its positions is correlated with the identity of the fluorophore attached to that nonamer. To the extent that the ligase discriminates for complementarily at that queried position, the fluorescent signal allows the inference of the identity of the base. After performing the ligation and four-color imaging, the anchor primer:nonamer complexes are stripped and a new cycle begins.

[00256] *Identify bins with non-maternal alleles (e.g. fetal cells)*: The first step in the data analysis procedure would be to use the 6bp DNA tags to sort the 200,000 sequence reads into bins which correspond to the individual wells of the microtiter plates. The ~400 sequence reads from each of the bins would then be separated into the different STR groups using standard sequence alignment algorithms. The aligned sequences from each of the bins would then be analyzed to identify non-maternal alleles. These can be identified in one of two ways. First, an independent blood sample fraction known to contain only maternal cells can be analyzed as described above. This sample can be a white blood cell fraction (which will contain only negligible numbers of fetal cells), or simply a dilution of the original sample before enrichment. Alternatively, the genotype profiles for all the wells can be similarity-clustered to identify the dominant pattern associated with maternal cells. In either approach, the detection of non-maternal alleles then determines which wells in the initial microtiter plate contained fetal cells. Determining the number bins with non-maternal alleles relative to the total number of bins provides an estimate of the number of fetal cells that were present in the original enriched cell population. Bins containing fetal cells would be identified with high levels of confidence because the non-maternal alleles are detected by multiple independent STRs.

[00257] *Detect ploidy for chromosomes 13, 18, and 21:* After identifying the bins that contained fetal cells, the next step would be to determine the ploidy of chromosomes 13, 18 and 21 by comparing the ratio of maternal to paternal alleles for each of the STRs. Again, for each bin there will be ~33 sequence reads for each of the 12 STRs. In a normal fetus, a given STR will have 1:1 ratio of the maternal to paternal alleles with approximately 16 sequence reads corresponding to each allele (normal diallelic). In a trisomic fetus, three doses of an STR marker can be detected either as three alleles with a 1:1:1 ratio (trisomic triallelic) or two alleles with a ratio of 2:1 (trisomic diallelic). In rare instances all three alleles may coincide and the locus will not be informative for that individual patient. The information from the different STRs on each chromosome can be combined to increase the confidence of a given aneuploidy call. In addition, the information from the independent bins containing fetal cells can also be combined to further increase the confidence of the call.

Example 9. Device Embodiment

[00258] Microfluidic devices of the invention were designed by computer-aided design (CAD) and microfabricated by photolithography. A two-step process was developed in which a blood sample is first debulked to remove the large population of small cells, and then the rare target epithelial cells target cells are recovered by immunoaffinity capture. The devices were defined by photolithography and etched into a silicon substrate based on the CAD-

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generated design. The cell enrichment module, which is approximately the size of a standard microscope slide, contains 14 parallel sample processing sections and associated sample handling channels that connect to common sample and buffer inlets and product and waste outlets. Each section contains an array of microfabricated obstacles that is optimized to enrich the target cell type by hydrodynamic size via displacement of the larger cells into the product stream. In this example, the microchip was designed to separate red blood cells (RBCs) and platelets from the larger leukocytes and CTCs. Enriched populations of target cells were recovered from whole blood passed through the device. Performance of the cell enrichment microchip was evaluated by separating RBCs and platelets from white blood cells (WBCs) in normal whole blood (**Figure 15**). In cancer patients, CTCs are found in the larger WBC fraction. Blood was minimally diluted (30%), and a 6 ml sample was processed at a flow rate of up to 6 ml/hr. The product and waste stream were evaluated in a Coulter Model "A^C-T diff" clinical blood analyzer, which automatically distinguishes, sizes, and counts different blood cell populations. The enrichment chip achieved separation of RBCs from WBCs, in which the WBC fraction had >99% retention of nucleated cells, >99% depletion of RBCs, and >97% depletion of platelets. Representative histograms of these cell fractions are shown in **Figure16**. Routine cytology confirmed the high degree of enrichment of the WBC and RBC fractions (**Figure 17**).

[00259] Next, epithelial cells were recovered by affinity capture in a microfluidic module that is functionalized with immobilized antibody. A capture module with a single chamber containing a regular array of antibody-coated microfabricated obstacles was designed. These obstacles are disposed to maximize cell capture by increasing the capture area approximately four-fold, and by slowing the flow of cells under laminar flow adjacent to the obstacles to increase the contact time between the cells and the immobilized antibody. The capture modules may be operated under conditions of relatively high flow rate but low shear to protect cells against damage. The surface of the capture module was functionalized by sequential treatment with 10% silane, 0.5% gluteraldehyde, and avidin, followed by biotinylated anti-EpCAM. Active sites were blocked with 3% bovine serum albumin in PBS, quenched with dilute Tris HCl, and stabilized with dilute L-histidine. Modules were washed in PBS after each stage and finally dried and stored at room temperature. Capture performance was measured with the human advanced lung cancer cell line NCI-H1650 (ATCC Number CRL-5883). This cell line has a heterozygous 15 bp in-frame deletion in exon 19 of EGFR that renders it susceptible to gefitinib. Cells from confluent cultures were harvested with trypsin, stained with the vital dye Cell Tracker Orange (CMRA reagent, Molecular Probes, Eugene, OR), resuspended in fresh whole blood, and fractionated in the microfluidic chip at various flow rates. In these initial feasibility experiments, cell suspensions were processed directly in the capture modules without prior fractionation in the cell enrichment module to debulk the red blood cells; hence, the sample stream contained normal blood red cells and leukocytes as well as tumor cells. After the cells were processed in the capture module, the device was washed with buffer at a higher flow rate (3ml/hr) to remove the nonspecifically bound cells. The adhesive top was removed and the adherent cells were fixed on the chip with paraformaldehyde and observed by fluorescence microscopy. Cell recovery was calculated from hemacytometer counts; representative capture results are shown in **Table 4**. Initial yields in reconstitution studies with unfractionated blood were greater than 60% with less than 5% of non-specific binding.

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Table 4

Run number	Avg. flow rate	Length of run	No. cells processed	No. cells captured	Yield
1	3.0	1 hr	150,000	36,012	25%
2	1.5	2 hr	150,000	30,000/ml	60%
3	1.08	2 hr	106,000	68,661	64%
4	1.21	2 hr	121,000	75,491	62%

[00260] Next, NCI-H1650 cells that were spiked into whole blood and recovered by size fractionation and affinity capture as described above were successfully analyzed in situ. In a trial run to distinguish epithelial cells from leukocytes, 0.5 ml of a stock solution of fluorescein-labeled CD45 pan-leukocyte monoclonal antibody were passed into the capture module and incubated at room temperature for 30 minutes. The module was washed with buffer to remove unbound antibody, and the cells were fixed on the chip with 1% paraformaldehyde and observed by fluorescence microscopy. As shown in **Figure 18**, the epithelial cells were bound to the obstacles and floor of the capture module. Background staining of the flow passages with CD45 pan-leukocyte antibody is visible, as are several stained leukocytes, apparently because of a low level of non-specific capture.

Example 10: Device embodiments

[00261] A design for preferred device embodiments of the invention is shown in **Figure 19A**, and parameters corresponding to three preferred device embodiments associated with this design are shown in **Figures 19B** and **19C**. These embodiments are particularly useful for enrich epithelial cells from blood.

Example 11: Determining counts for large cell types

[00262] Using the methods of the invention, a diagnosis of the absence, presence, or progression of cancer may be based on the number of cells in a cellular sample that are larger than a particular cutoff size. For example, cells with a hydrodynamic size of 14 microns or larger may be selected. This cutoff size would eliminate most leukocytes. The nature of these cells may then be determined by downstream molecular or cytological analysis.

[00263] Cell types other than epithelial cells that would be useful to analyze include endothelial cells, endothelial progenitor cells, endometrial cells, or trophoblasts indicative of a disease state. Furthermore, determining separate counts for epithelial cells, e.g., cancer cells, and other cell types, e.g., endothelial cells, followed by a determination of the ratios between the number of epithelial cells and the number of other cell types, may provide useful diagnostic information.

[00264] A device of the invention may be configured to isolate targeted subpopulations of cells such as those described above, as shown in **Figures 20A-D**. A size cutoff may be selected such that most native blood cells, including red blood cells, white blood cells, and platelets, flow to waste, while non-native cells, which could include endothelial cells, endothelial progenitor cells, endometrial cells, or trophoblasts, are collected in an enriched sample. This enriched sample may be further analyzed.

[00265] Using a device of the invention, therefore, it is possible to isolate a subpopulation of cells from blood or other bodily fluids based on size, which conveniently allows for the elimination of a large proportion of native blood cells when large cell types are targeted. As shown schematically in **Figure 21**, a device of the invention may include counting means to determine the number of cells in the enriched sample, or the number of cells of a particular type, e.g., cancer cells, within the enriched sample, and further analysis of the cells in the enriched sample may provide additional information that is useful for diagnostic or other purposes.

Example 12: Method for detection of EGFR mutations

[00266] A blood sample from a cancer patient is processed and analyzed using the devices and methods of the invention, resulting in an enriched sample of epithelial cells containing CTCs. This sample is then analyzed to

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identify potential EGFR mutations. The method permits both identification of known, clinically relevant EGFR mutations as well as discovery of novel mutations. An overview of this process is shown in **Figure 22**.

[00267] Below is an outline of the strategy for detection and confirmation of EGFR mutations:

1) Sequence CTC EGFR mRNA

a) Purify CTCs from blood sample;

b) Purify total RNA from CTCs;

c) Convert RNA to cDNA using reverse transcriptase;

d) Use resultant cDNA to perform first and second PCR reactions for generating sequencing templates; and

e) Purify the nested PCR amplicon and use as a sequencing template to sequence EGFR exons 18-21.

2) Confirm RNA sequence using CTC genomic DNA

a) Purify CTCs from blood sample;

b) Purify genomic DNA (gDNA) from CTCs;

c) Amplify exons 18, 19, 20, and/or 21 via PCR reactions; and

d) Use the resulting PCR amplicon(s) in real-time quantitative allele-specific PCR reactions in order to confirm the sequence of mutations discovered via RNA sequencing.

[00268] Further details for each step outlined above are as follows.

1) Sequence CTC EGFR mRNA

a) Purify CTCs from blood sample. CTCs are isolated using any of the size-based enrichment and/or affinity purification devices of the invention.

b) Purify total RNA from CTCs. Total RNA is then purified from isolated CTC populations using, e.g., the Qiagen Micro RNeasy kit, or a similar total RNA purification protocol from another manufacturer; alternatively, standard RNA purification protocols such as guanidium isothiocyanate homogenization followed by phenol/chloroform extraction and ethanol precipitation may be used. One such method is described in "Molecular Cloning – A Laboratory Manual, Second Edition" (1989) by J. Sambrook, E.F. Fritch and T. Maniatis, p. 7.24.

c) Convert RNA to cDNA using reverse transcriptase. cDNA reactions are carried out based on the protocols of the supplier of reverse transcriptase. Typically, the amount of input RNA into the cDNA reactions is in the range of 10 picograms (pg) to 2 micrograms (μg) total RNA. First-strand DNA synthesis is carried out by hybridizing random 7mer DNA primers, or oligo-dT primers, or gene-specific primers, to RNA templates at 65°C followed by snap-chilling on ice. cDNA synthesis is initiated by the addition of iScript Reverse Transcriptase (BioRad) or SuperScript Reverse Transcriptase (Invitrogen) or a reverse transcriptase from another commercial vendor along with the appropriate enzyme reaction buffer. For iScript, reverse transcriptase reactions are carried out at 42°C for 30-45 minutes, followed by enzyme inactivation for 5 minutes at 85°C. cDNA is stored at -20°C until use or used immediately in PCR reactions. Typically, cDNA reactions are carried out in a final volume of 20 μl, and 10% (2 μl) of the resultant cDNA is used in subsequent PCR reactions.

d) Use resultant cDNA to perform first and second PCR reactions for generating sequencing templates. cDNA from the reverse transcriptase reactions is mixed with DNA primers specific for the region of interest (**Figure 23**). See **Table 5** for sets of primers that may be used for amplification of exons 18-21. In **Table 5**, primer set M13(+)/M12(-) is internal to primer set M11(+)/M14(-). Thus primers M13(+) and M12(-) may be used in the nested round of amplification, if primers M11(+) and M14(-) were used in the first round of expansion. Similarly, primer set M11(+)/M14(-) is internal to primer set M15(+)/M16(-), and primer set

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M23(+)/M24(-) is internal to primer set M21(+)/M22(-). Hot Start PCR reactions are performed using Qiagen HotStar Taq Polymerase kit, or Applied Biosystems HotStart TaqMan polymerase, or other Hot Start thermostable polymerase, or without a hot start using Promega GoTaq Green Taq Polymerase master mix, TaqMan DNA polymerase, or other thermostable DNA polymerase. Typically, reaction volumes are 50 µl, nucleotide triphosphates are present at a final concentration of 200 µM for each nucleotide, $MgCl_2$ is present at a final concentration of 1-4 mM, and oligo primers are at a final concentration of 0.5 µM. Hot start protocols begin with a 10-15 minute incubation at 95°C, followed by 40 cycles of 94°C for one minute (denaturation), 52°C for one minute (annealing), and 72°C for one minute (extension). A 10 minute terminal extension at 72°C is performed before samples are stored at 4°C until they are either used as template in the second (nested) round of PCRs, or purified using QiaQuick Spin Columns (Qiagen) prior to sequencing. If a hot-start protocol is not used, the initial incubation at 95°C is omitted. If a PCR product is to be used in a second round of PCRs, 2 µl (4%) of the initial PCR product is used as template in the second round reactions, and the identical reagent concentrations and cycling parameters are used.

Table 5. Primer Sets for expanding EGFR mRNA around Exons 18-21

Name	SEQ ID NO	Sequence (5' to 3')	cDNA Coordinates	Amplicon Size
NXK-M11(+)	1	TTGCTGCTGGTGGTGGC	(+) 1966-1982	813
NXK-M14(-)	2	CAGGGATTCCGTCATATGGC	(-) 2778-2759	
NXK-M13(+)	3	GATCGGCCTCTTCATGCG	(+) 1989-2006	747
NXK M12(-)	4	GATCCAAAGGTCATCAACTCCC	(-) 2735-2714	
NXK-M15(+)	5	GCTGTCCAACGAATGGGC	(+) 1904-1921	894
NXK-M16(-)	6	GGCGTTCTCCTTTCTCCAGG	(-) 2797-2778	
NXK-M21(+)	7	ATGCACTGGGCCAGGTCTT	(+) 1881-1899	944
NXK-M22(-)	8	CGATGGTACATATGGGTGGCT	(-) 2824-2804	
NXK-M23(+)	9	AGGCTGTCCAACGAATGGG	(+) 1902-1920	904
NXK-M24(-)	10	CTGAGGGAGGCGTTCTCCT	(-) 2805-2787	

e) *Purify* the *nested PCR amplicon and use as a sequencing template to sequence EGFR exons 18-21.* Sequencing is performed by ABI automated fluorescent sequencing machines and fluorescence-labeled DNA sequencing ladders generated via Sanger-style sequencing reactions using fluorescent dideoxynucleotide mixtures. PCR products are purified using Qiagen QuickSpin columns, the Agencourt AMPure PCR Purification System, or PCR product purification kits obtained from other vendors. After PCR products are purified, the nucleotide concentration and purity is determined with a Nanodrop 7000 spectrophotometer, and the PCR product concentration is brought to a concentration of 25 ng/µl. As a quality control measure, only PCR products that have a UV-light absorbance ratio (A_{260}/A_{280}) greater than 1.8 are used for sequencing. Sequencing primers are brought to a concentration of 3.2 pmol/µl.

2) Confirm RNA sequence using CTC genomic DNA

a) Purify *CTCs from blood sample.* As above, CTCs are isolated using any of the size-based enrichment and/or affinity purification devices of the invention.

b) *Purify genomic DNA (gDNA) from CTCs.* Genomic DNA is purified using the Qiagen DNeasy Mini kit, the Invitrogen ChargeSwitch gDNA kit, or another commercial kit, or via the following protocol:

1. Cell pellets are either lysed fresh or stored at -80°C and are thawed immediately before lysis.

2. Add 500 µl 50mM Tris pH 7.9/100mM EDTA/0.5%SDS (TES buffer).

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3. Add 12.5 µl Proteinase K (IBI5406, 20mg/ml), generating a final [ProtK] = 0.5 mg/ml.

4. Incubate at 55°C overnight in rotating incubator.

5. Add 20 µl of RNase cocktail (500 U/ml RNase A + 20,000 U/ml RNase T1, Ambion #2288) and incubate four hours at 37°C.

6. Extract with Phenol (Kodak, Tris pH 8 equilibrated), shake to mix, spin 5 min. in tabletop centrifuge.

7. Transfer aqueous phase to fresh tube.

8. Extract with Phenol/Chloroform/Isoamyl alcohol (EMD, 25:24:1 ratio, Tris pH 8 equilibrated), shake to mix, spin five minutes in tabletop centrifuge.

9. Add 50 µl 3M NaOAc pH = 6.

10. Add 500 µl EtOH.

11. Shake to mix. Strings of precipitated DNA may be visible. If anticipated DNA concentration is very low, add carrier nucleotide (usually yeast tRNA).

12. Spin one minute at max speed in tabletop centrifuge.

13. Remove supernatant.

14. Add 500 µl 70% EtOH, Room Temperature (RT)

15. Shake to mix.

16. Spin one minute at max speed in tabletop centrifuge.

17. Air dry 10-20 minutes before adding TE.

18. Resuspend in 400 µl TE. Incubate at 65°C for 10 minutes, then leave at RT overnight before quantitation on Nanodrop.

c) Amplify exons 18, 19, 20, and/or 21 via PCR reactions. Hot start nested PCR amplification is carried out as described above in step **1d**, except that there is no nested round of amplification. The initial PCR step may be stopped during the log phase in order to minimize possible loss of allele-specific information during amplification. The primer sets used for expansion of EGFR exons 18-21 are listed in **Table 6** (see also Paez et al., Science 304:1497-1500 (Supplementary Material) (2004)).

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Table 6. Primer sets for expanding EGFR genomic DNA

Name	SEQ ID NO	Sequence (5' to 3')	Exon	Amplicon Size
NXK-ex18.1(+)	11	TCAGAGCCTGTGTTTCTACCAA	18	534
NXK-ex18.2(-)	12	TGGTCTCACAGGACCACTGATT	18	
NXK-ex18.3(+)	13	TCCAAATGAGCTGGCAAGTG	18	397
NXK-ex18.4(-)	14	TCCCAAACACTCAGTGAAACAAA	18	
NXK-ex19.1(+)	15	AAATAATCAGTGTGATTCGTGGAG	19	495
NXK-ex19.2(-)	16	GAGGCCAGTGCTGTCTCTAAGG	19	
NXK-ex19.3(+)	17	GTGCATCGCTGGTAACATCC	19	298
NXK-ex19.4(-)	18	TGTGGAGATGAGCAGGGTCT	19	
NXK-ex20.1(+)	19	ACTTCACAGCCCTGCGTAAAC	20	555
NXK-ex20.2(-)	20	ATGGGACAGGCACTGATTTGT	20	
NXK-ex20.3(+)	21	ATCGCATTCATGCGTCTTCA	20	379
NXK-ex20.4(-)	22	ATCCCCATGGCAAACTCTTG	20	
NXK-ex21.1(+)	23	GCAGCGGGTTACATCTTCTTTC	21	526
NXK-ex21.2(-)	24	CAGCTCTGGCTCACACTACCAG	21	
NXK-ex21.3(+)	25	GCAGCGGGTTACATCTTCTTTC	21	349
NXK-ex21.4(-)	26	CATCCTCCCCTGCATGTGT	21	

d) Use the resulting PCR amplicon(s) in real-time quantitative allele-specific PCR reactions in order to confirm the sequence of mutations discovered via RNA sequencing. An aliquot of the PCR amplicons is used as template in a multiplexed allele-specific quantitative PCR reaction using TaqMan PCR 5' Nuclease assays with an Applied Biosystems model 7500 Real Time PCR machine (**Figure 24**). This round of PCR amplifies subregions of the initial PCR product specific to each mutation of interest. Given the very high sensitivity of Real Time PCR, it is possible to obtain complete information on the mutation status of the EGFR gene even if as few as 10 CTCs are isolated. Real Time PCR provides quantification of allelic *sequences* over 8 logs of input DNA concentrations; thus, even heterozygous mutations in impure populations are easily detected using this method.

[00269] Probe and primer sets are designed for all known mutations that affect gefitinib responsiveness in NSCLC patients, including over 40 such somatic mutations, including point mutations, deletions, and insertions, that have been reported in the medical literature. For illustrative purposes, examples of primer and probe sets for five of the point mutations are listed in **Table 7**. In general, oligonucleotides may be designed using the primer optimization software program Primer Express (Applied Biosystems), with hybridization conditions optimized to distinguish the wild type EGFR DNA sequence from mutant alleles. EGFR genomic DNA amplified from lung cancer cell lines that are known to carry EGFR mutations, such as H358 (wild type), H1650 (15-bp deletion, Δ2235-2249), and H1975 (two point mutations, 2369 C→ T, 2573 T→ G), is used to optimize the allele-specific Real Time PCR reactions. Using the TaqMan 5' nuclease assay, allele-specific labeled probes specific for wild type sequence or for known EGFR mutations are developed. The oligonucleotides are designed to have melting temperatures that easily distinguish a match from a mismatch, and the Real Time PCR conditions are optimized to distinguish wild type and mutant alleles. All Real Time PCR reactions are carried out in triplicate.

[00270] Initially, labeled probes containing wild type sequence are multiplexed in the same reaction with a single mutant probe. Expressing the results as a ratio of one mutant allele sequence versus wild type sequence may identify samples containing or lacking a given mutation. After conditions are optimized for a given probe set, it is then possible to multiplex probes for all of the mutant alleles within a given exon within the same Real Time PCR assay, increasing the ease of use of this analytical tool in clinical settings.

[00271] A unique probe is designed for each wild type allele and mutant allele sequence. Wild-type sequences are marked with the fluorescent dye VIC at the 5' end, and mutant sequences with the fluorophore FAM. A

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fluorescence quencher and Minor Groove Binding moiety are attached to the 3' ends of the probes. ROX is used as a passive reference dye for normalization purposes. A standard curve is generated for wild type sequences and is used for relative quantitation. Precise quantitation of mutant signal is not required, as the input cell population is of unknown, and varying, purity. The assay is set up as described by ABI product literature, and the presence of a mutation is confirmed when the signal from a mutant allele probe rises above the background level of fluorescence (**Figure 25**), and this threshold cycle gives the relative frequency of the mutant allele in the input sample.

Table 7. Probes and Primers for Allele-Specific qPCR

Name	SEQ ID NO	Sequence (5' to 3', mutated position in bold)	EMBL Chromosome 7 Genomic Coordinates	Description	Mut
NXK-M01	27	CCGCAGCATGTCAAGATCAC	(+)55,033,694-55,033,713	(+) primer	
NXK-M02	28	TCCTTCTGCATGGTATTCTTTCTCT	(-)55,033,769-55,033,745	(-) primer	
Pwt-L858R	29	VIC-TTTGGGCTGGCCAA-MGB	(+)55,033,699-55,033,712	WT allele probe	L858
Pmut-L858R	30	FAM-TTTTGGGCGGGCCA-MGB	(+)55,033,698-55,033,711	Mutant allele probe	
NXK-M03	31	ATGGCCAGCGTGGACAA	(+)55,023,207-55,023,224	(+) primer	
NXK-M04	32	AGCAGGTACTGGGAGCCAATATT	(-)55,023,355-55,023,333	(-) primer	
Pwt-T790M	33	VIC-ATGAGCTGCGTGATGA-MGB	(-)55,023,290-55,023,275	WT allele probe	T790
Pmut-T790M	34	FAM-ATGAGCTGCATGATGA-MGB	(-)55,023,290-55,023,275	Mutant allele probe	
NXK-M05	35	GCCTCTTACACCCAGTGGAGAA	(+)55,015,831-55,015,852	(+) primer	
NXK-ex18.5	36	GCCTGTGCCAGGGACCTT	(-)55,015,965-55,015,948	(-) primer	
Pwt-G719SC	37	VIC-ACCGGAGCCCAGCA-MGB	(-)55,015,924-55,015,911	WT allele probe	G71
Pmut-G719S	38	FAM-ACCGGAGCTCAGCA-MGB	(-)55,015,924-55,015,911	Mutant allele probe	
mut-G719C	39	FAM-ACCGGAGCACAGCA-MGB	(-)55,015,924-55,015,911	Mutant allele probe	
NXK-ex21.5	40	ACAGCAGGGTCTTCTCTGTTTCAG	(+)55,033,597-55,033,620	(+) primer	
NXK-M10	41	ATCTTGACATGCTGCGGTGTT	(-)55,033,710 55,033,690	(-) primer	
Pwt-H835L	42	VIC-TTGGTGCACCGCGA-MGB	(+)55,033,803-55,033,816	WT allele probe	H8
Pmut-H835L	43	FAM-TGGTGCTCCGCGAC-MGB	(+)55,033,803-55,033,816	Mutant allele probe	
NXK-M07	101	TGGATCCCAGAAGGTGAGAAA	(+)55,016,630-55,016,650	(+) primer	
NXK-ex19.5	102	AGCAGAAACTCACATCGAGGATTT	(-)55,016,735-55,016,712	(-) primer	
Pwt-delE746-A750	103	AAGGAATTAAGAGAAGCAA	(+)55,016,681-55,016,699	WT allele probe	delE A7
Pmut-delE746-A750var1	104	CTATCAAAACATCTCC	(+)55,016,676-55,016,691	Mutant allele probe, variant 1	
Pmut-delE746-A750var1	105	CTATCAAGACATCTCC	(+)55,016,676-55,016,691	Mutant allele probe, variant 2	

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Example 13: Absence of EGFR expression in leukocytes

[00272] To test whether EGFR mRNA is present in leukocytes, several PCR experiments were performed. Four sets of primers, shown in **Table 8**, were designed to amplify four corresponding genes:

1) BCKDK (branched-chain a-ketoacid dehydrogenase complex kinase) – a "housekeeping" gene expressed in all types of cells, a positive control for both leukocytes and tumor cells;

2) CD45 – specifically expressed in leukocytes, a positive control for leukocytes and a negative control for tumor cells;

3) EpCaM – specifically expressed in epithelial cells, a negative control for leukocytes and a positive control for tumor cells; and

4) EGFR – the target mRNA to be examined.

Table 8

Name	SEQ ID NO	Sequence (5' to 3')	Description	Amplicon Size
BCKD_1	44	AGTCAGGACCCATGCACGG	BCKDK (+) primer	273
BCKD_2	45	ACCCAAGATGCAGCAGTGTG	BCKDK (-) primer	
CD45_1	46	GATGTCCTCCTTGTTCTACTC	CD45 (+) primer	263
CD45_2	47	TACAGGGAATAATCGAGCATGC	CD45 (-) primer	
EpCAM_1	48	GAAGGGAAATAGCAAATGGACA	EpCAM (+) primer	222
EpCAM_2	49	CGATGGAGTCCAAGTTCTGG	EpCAM (-) primer	
EGFR_1	50	AGCACTTACAGCTCTGGCCA	EGFR (+) primer	371
EGFR_2	51	GACTGAACATAACTGTAGGCTG	EGFR (-) primer	

[00273] Total RNAs of approximately 9×10^6 leukocytes isolated using a cell enrichment device of the invention (cutoff size 4 μm) and 5×10^5 H1650 cells were isolated by using RNeasy mini kit (Qiagen). Two micrograms of total RNAs from leukocytes and H1650 cells were reverse transcribed to obtain first strand cDNAs using 100 pmol random hexamer (Roche) and 200 U Superscript II (Invitrogen) in a 20 μl reaction. The subsequent PCR was carried out using 0.5 μl of the first strand cDNA reaction and 10 pmol of forward and reverse primers in total 25 μl of mixture. The PCR was run for 40 cycles of 95°C for 20 seconds, 56°C for 20 seconds, and 70°C for 30 seconds. The amplified products were separated on a 1% agarose gel. As shown in **Figure 26A**, BCKDK was found to be expressed in both leukocytes and H1650 cells; CD45 was expressed only in leukocytes; and both EpCAM and EGFR were expressed only in H1650 cells. These results, which are fully consistent with the profile of EGFR expression shown in **Figure 26B**, confirmed that EGFR is a particularly useful target for assaying mixtures of cells that include both leukocytes and cancer cells, because only the cancer cells will be expected to produce a signal.

Example 14: EGFR assay with low quantities of target RNA or high quantities of background RNA

[00274] In order to determine the sensitivity of the assay described in **Example 12**, various quantities of input NSCLC cell line total RNA were tested, ranging from 100 pg to 50 ng. The results of the first and second EGFR PCR reactions (**step 1d, Example 12**) are shown in **Figure 27**. The first PCR reaction was shown to be sufficiently sensitive to detect 1 ng of input RNA, while the second round increased the sensitivity to 100 pg or less of input RNA. This corresponds to 7-10 cells, demonstrating that even extremely dilute samples may generate detectable signals using this assay.

[00275] Next, samples containing 1 ng of NCI-H1975 RNA were mixed with varying quantities of peripheral blood mononuclear cell (PBMC) RNA ranging from 1 ng to 1 μg and used in PCR reactions as before. As shown in **Figure 28A**, the first set of PCR reactions demonstrated that, while amplification occurred in all cases, spurious bands appeared at the highest contamination level. However, as shown in **Figure 28B**, after the second, nested set

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of PCR reactions, the desired specific amplicon was produced without spurious bands even at the highest contamination level. Therefore, this example demonstrates that the EGFR PCR assays described herein are effective even when the target RNA occupies a tiny fraction of the total RNA in the sample being tested.

[00276] **Table 8** lists the RNA yield in a variety of cells and shows that the yield per cell is widely variable, depending on the cell type. This information is useful in order to estimate the amount of target and background RNA in a sample based on cell counts. For example, 1 ng of NCL-H1975 RNA corresponds to approximately 100 cells, while 1 μg of PBMC RNA corresponds to approximately 10^6 cells. Thus, the highest contamination level in the above-described experiment, 1,000:1 of PBMC RNA to NCL-H1975 RNA, actually corresponds to a 10,000:1 ratio of PBMCs to NCL-H1975 cells. Thus, these data indicate that EGFR may be sequenced from as few as 100 CTCs contaminated by as many as 10^6 leukocytes.

Table 8. RNA Yield versus Cell Type

Cells	Count	RNA Yield	[RNA]/Cell
NCI-H1975	2×10^6	26.9 μg	13.5 pg
NCI-H1650	2×10^6	26.1 μg	13.0 pg
H358	2×10^6	26.0 μg	13.0 pg
HT29	2×10^6	21.4 μg	10.7 pg
MCF7	2×10^6	25.4 μg	12.7 pg
PBMC #1	19×10^6	10.2 μg	0.5 pg
PBMC #2	16.5×10^6	18.4 μg	1.1 pg

[00277] Next, whole blood spiked with 1,000 cells/ml of Cell Tracker (Invitrogen)-labeled H1650 cells was run through the capture module chip of Figure 19C. To avoid inefficiency in RNA extraction from fixed samples, the captured H1650 cells were immediately counted after running and subsequently lysed for RNA extraction without formaldehyde fixation. Approximately 800 captured H1650 cells and >10,000 contaminated leukocytes were lysed on the chip with 0.5 ml of 4M guanidine thiocyanate solution. The lysate was extracted with 0.5 ml of phenol/chloroform and precipitated with 1ml of ethanol in the presence of 10 μg of yeast tRNA as carrier. The precipitated RNAs were DNase I-treated for 30 minutes and then extracted with phenol/chloroform and precipitated with ethanol prior to first strand cDNA synthesis and subsequent PCR amplification. These steps were repeated with a second blood sample and a second chip. The cDNA synthesized from chip1 and chip2 RNAs along with H1650 and leukocyte cDNAs were PCR amplified using two sets of primers, CD45_1 and CD45_2 (**Table 7**) as well as EGFR_5 (forward primer, 5'-GTTCGGCACGGTGTATAAGG-3') (SEQ ID NO: ___) and EGFR_6 (reverse primer, 5'-CTGGCCATCACGTAGGCTTC-3') (SEQ ID NO: ___). EGFR_5 and EGFR_6 produce a 138 bp wild type amplified fragment and a 123 bp mutant amplified fragment in H1650 cells. The PCR products were separated on a 2.5% agarose gel. As shown in **Figure 29**, EGFR wild type and mutant amplified fragments were readily detected, despite the high leukocyte background, demonstrating that the EGFR assay is robust and does not require a highly purified sample.

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WHAT IS CLAIMED IS:

1. A method for fetal diagnosis comprising:

labeling one or more genomic DNA regions in fetal and non-fetal cells, enriched from a maternal blood sample, using labels adapted to distinguish between individual cells, and

determining the presence or absence of a fetal abnormality by analyzing said labeled genomic DNA regions.

2. A method for fetal diagnosis comprising:

labeling one or more cDNA sequences generated from fetal and non-fetal cells, wherein said fetal and non-fetal cells are enriched from a maternal blood sample, using labels adapted to distinguish between individual cells, and

determining the presence or absence of a fetal abnormality by analyzing said labeled cDNA regions.

3. The method of claim 1, further comprising splitting said fetal and non-fetal cells such that individual cells are in addressable locations.

4. The method of claim 2,further comprising splitting said fetal and non-fetal cells such that individual cells are in addressable locations.

5. The method of claim 3, further comprising amplifying said one or more genomic DNA regions from each cell to generate a plurality of amplicons.

6. . The method of claim 5, further comprising pooling said amplicons.

7. The method of claim 5 wherein said regions of genomic DNA comprise one or more polymorphisms.

8. The method of claim 7, wherein said polymorphisms are STRs or SNPs.

9. The method of claim 5 wherein said amplification is performed by multiplex PCR.

10. The method of claim 9 wherein said amplification also comprises nested PCR.

11. The method of claim 3, wherein said labels each comprise a unique tag sequence specific for each addressable location.

12. The method of claim 1 or 2 wherein said label comprises a nucleic acid sequence between 4-20 bp long that is unique to each cell

13. The method of claim 1, wherein at least two genomic DNA regions on a chromosome of interest are labeled.

14. The method of claim 1, wherein said genomic DNA regions are located on two or more chromosomes of interest.

15. The method of claim 1 or 2, wherein said analyzing comprises performing ultra deep sequencing or quantitative genotyping.

16. The method of claim 1 or 2, wherein said fetal abnormality comprises an aneuploidy selected from the group consisting of: trisomy 13, trisomy 18, trisomy 21 (Down Syndrome), Klinefelter Syndrome (XXY),

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monosomy, triploidy, tetraploidy, or other irregular number of sex or autosomal chromosomes, and a combination thereof.

17. The method of claim 1 or 2, wherein said fetal abnormality comprises an aneuploidy that is maternally or paternally derived.

18. The method of claim 1 or 2, wherein the fetal abnormality is a segmental aneuploidy.

19. The method of claim 15, wherein said quantitative genotyping is performed by using molecular inversion probe technology.

20. A method for fetal diagnosis comprising:

amplifying a plurality of genomic DNA regions from one or more fetal cells enriched from maternal blood; wherein the resulting amplicons comprise a cell specific label, and

determining the presence or absence of a fetal abnormality by analyzing said amplicons.

21. A method for fetal diagnosis comprising:

amplifying a plurality of cDNA sequence generated from one or more fetal cells enriched from maternal blood; wherein the resulting amplicons comprise a cell specific label, and

determining the presence or absence of a fetal abnormality by analyzing said amplicons.

22. The method of claim 20 or 21, further comprising splitting said one or more fetal cells such that each individual cell is in an addressable location.

23. The method of claim 20 or 21, further comprising pooling said amplicons.

24. The method of claim 20 or 21, wherein said regions of genomic DNA comprise one or more polymorphisms.

25. The method of claim 24, wherein said polymorphisms are STRs or SNPs.

26. The method of claim 20 or 21, wherein said amplification is performed by multiplex PCR, singleplex PCR, quantitative PCR, quantitative fluorescent PCR (QF-PCR), multiplex fluorescent PCR (MF-PCR), real time PCR (RT-PCR), single cell PCR, restriction fragment length polymorphism PCR (PCR-RFLP), PCR-RFLP/RT-PCR-RFLP, hot start PCR, in situ polonony PCR, in situ rolling circle amplification (RCA), bridge PCR, picotiter PCR, emulsion PCR, ligase chain reaction (LCR), transcription amplification, self-sustained sequence replication, selective amplification of target polynucleotide sequences, consensus sequence primed polymerase chain reaction (CP-PCR), arbitrarily primed polymerase chain reaction (AP-PCR), degenerate oligonucleotide-primed PCR (DOP-PCR), MDA and nucleic acid based sequence amplification (NABSA).

27. The method of claim 26 wherein said amplification also comprises nested PCR.

28. The method of claim 20 or 21, wherein said labels each comprise a unique tag sequence specific for each addressable location.

29. The method of claim 20 or 21, wherein said label comprises a nucleic acid sequence between 4-20 bp long that is unique to each cell

30. The method of claim 20 wherein at least two genomic DNA regions on a chromosome of interest are labeled.

31. The method of claim 20 wherein said genomic DNA regions are located on two or more chromosomes of interest.

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32. The method of claim 20 or 21, wherein said analyzing comprises performing ultra deep sequencing or quantitative genotyping.

33. The method of claim 20 or 21, wherein said fetal abnormality comprises an aneuploidy is selected from the group consisting of: trisomy 13, trisomy 18, trisomy 21 (Down Syndrome), Klinefelter Syndrome (XXY), other irregular number of sex or autosomal chromosomes, and a combination thereof.

34. The method of claim 20 or 21, wherein said fetal abnormality comprises an aneuploidy that is maternally or paternally derived.

35. The method of claim 20 or 21, wherein the fetal abnormality is a segmental aneuploidy.

36. The method of claim 32, wherein said quantitative genotyping is performed by using molecular inversion probe technology.

37. A computer readable medium comprising logic for analyzing a plurality of amplicons, from one or more genomic DNA regions on one or more chromosomes from at least one fetal cell and at least one maternal cell, enriched from maternal blood.

38. A computer readable medium comprising logic for analyzing a plurality of amplicons, from one or more cDNA sequences generated from at least one fetal cell and at least one maternal cell, enriched from maternal blood.

39. The computer readable medium of claim 37 or 39, wherein said logic analyzes the ratio of maternal to fetal chromosome copy number.

40. The method of claim 37 or 39, wherein said amplicons comprise a unique tag sequence

41. The method of claim 37 or 39, wherein said amplicons comprise one or more polymorphisms.

42. The method of claim 41, wherein said polymorphisms are STRs or SNPs.

43. The method of claim 37, wherein said plurality of amplicons are from at least two genomic DNA regions on a chromosome of interest.

44. The method of claim 37, wherein said plurality of amplicons are from genomic DNA regions located on two or more chromosomes of interest.

45. The method of claim 37 or 39, wherein said plurality of amplicons comprises at least 6,000 amplicons.

46. The method of claim 1, 2, 20 or 21, further comprising binning at least a portion of said enriched cells

47. The method of claim 46, wherein said binning is performed using a platform selected from the group consisting of: microtiter plates, PCR wells, a nanofludic system, microencapsulation and a combination thereof.

48. The method of claim 46, wherein said binning is performed using serial dilution.

49. The method of claim 46, further comprising to negative selection of non-target cells or positive selection of target cells, prior to said binning.

50. The method of claim 46 wherein said binning is performed using a number of bins less than 100.

51. The method of claim 46 wherein said binning is performed using a number of bins greater than 100.

52. The methods of claim 50 wherein said bins are analyzed for the presence of at least one fetal cell.

53. The methods of claim 51 wherein said bins are analyzed for the presence of at least one fetal cell.

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54. The method of claim 52 wherein said analysis is performed using an analytical method selected from the group consisting of: FISH, fetal biomarker labels, SNP detection, PCR, and STR detection.

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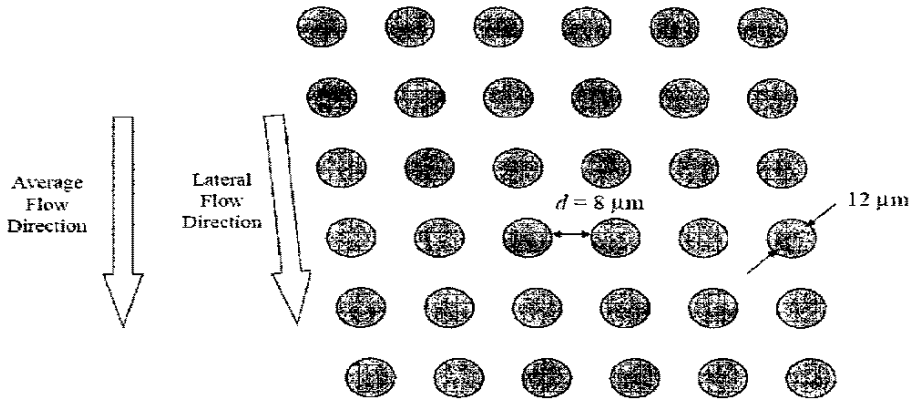


Figure 1A

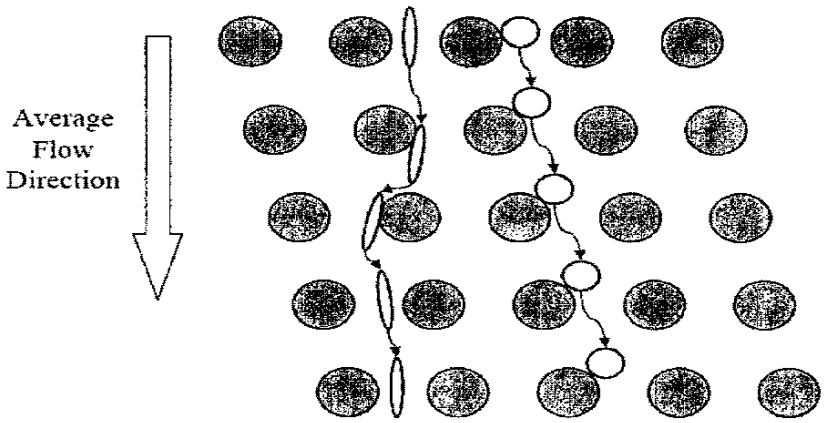


Figure 1B

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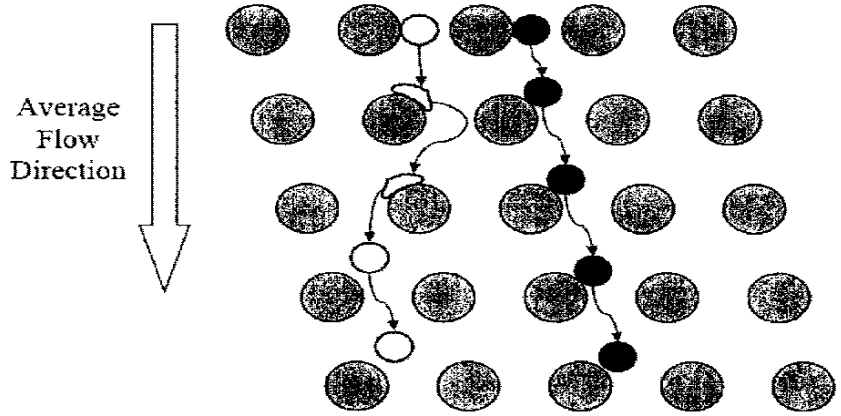


Figure 1C

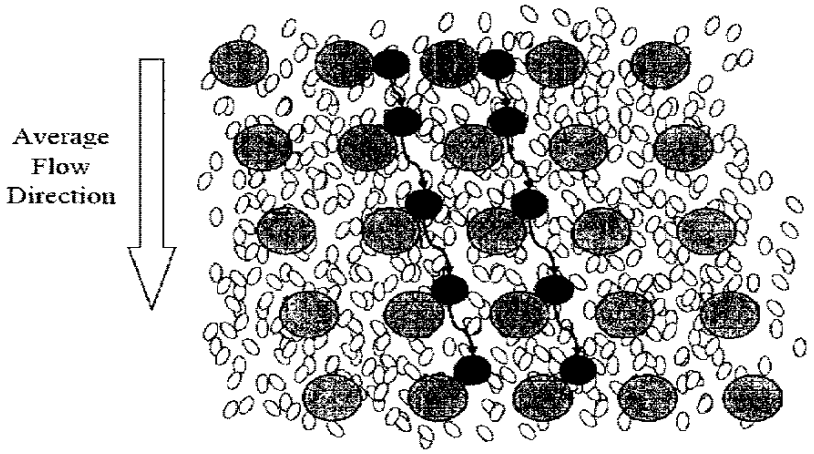


Figure 1D

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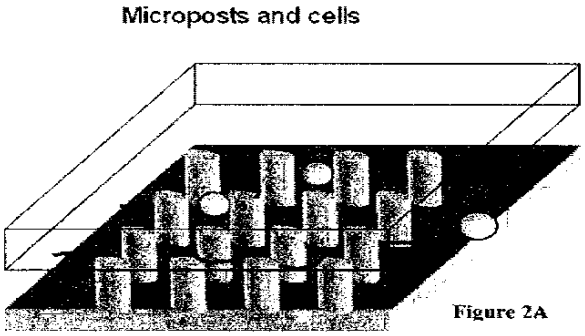


Figure 2A



Figure 2B

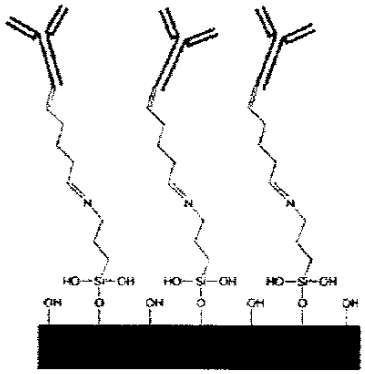


Figure 2C

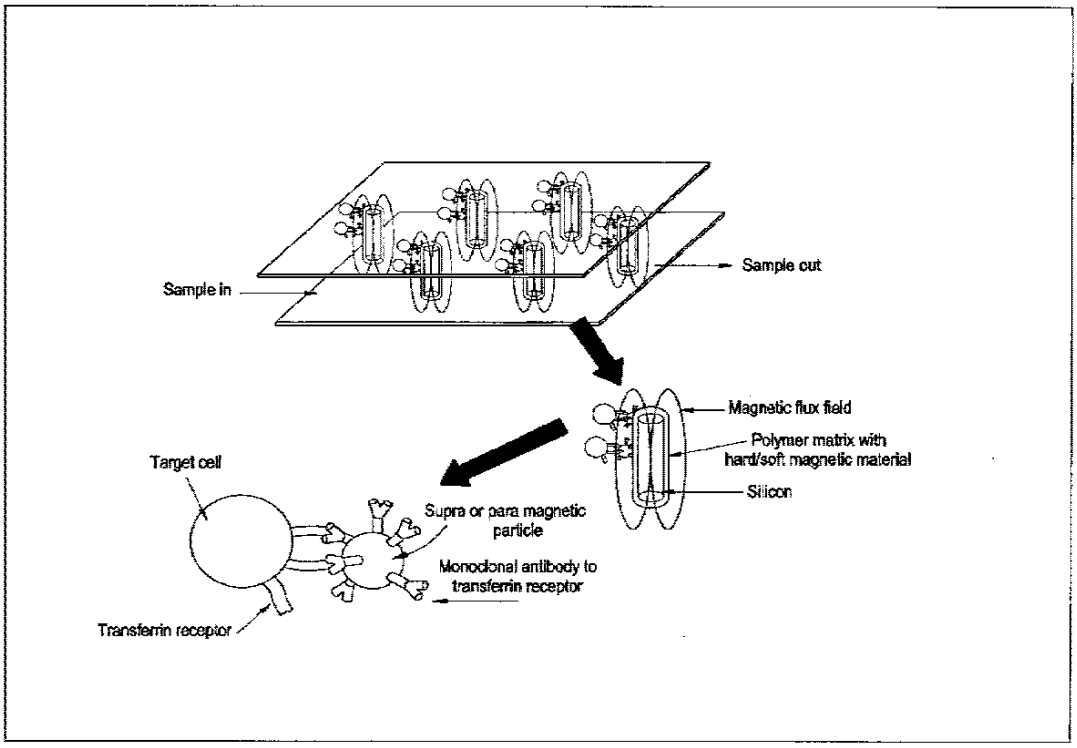

Sample in
Sample out
Magnetic flux field
Polymer matrix with
hard/soft magnetic material
Silicon
Target cell
Supra or para magnetic
particle
Monoclonal antibody to
transferrin receptor
Transferrin receptor

Figure 3

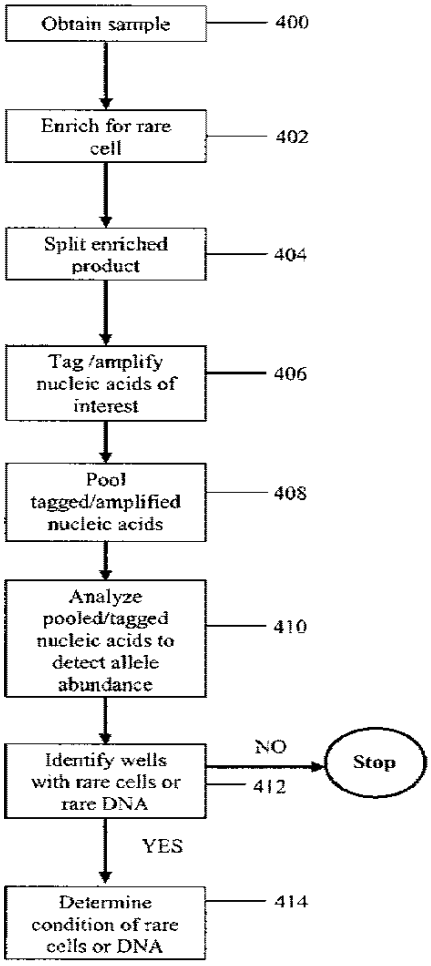

Obtain sample
400
Enrich for rare cell
402
Split enriched product
404
Tag /amplify nucleic acids of interest
406
Pool tagged/amplified nucleic acids
408
Analyze pooled/tagged nucleic acids to detect allele abundance
410
Identify wells with rare cells or rare DNA
NO
Stop
412
YES
Determine condition of rare cells or DNA
414

Figure 4

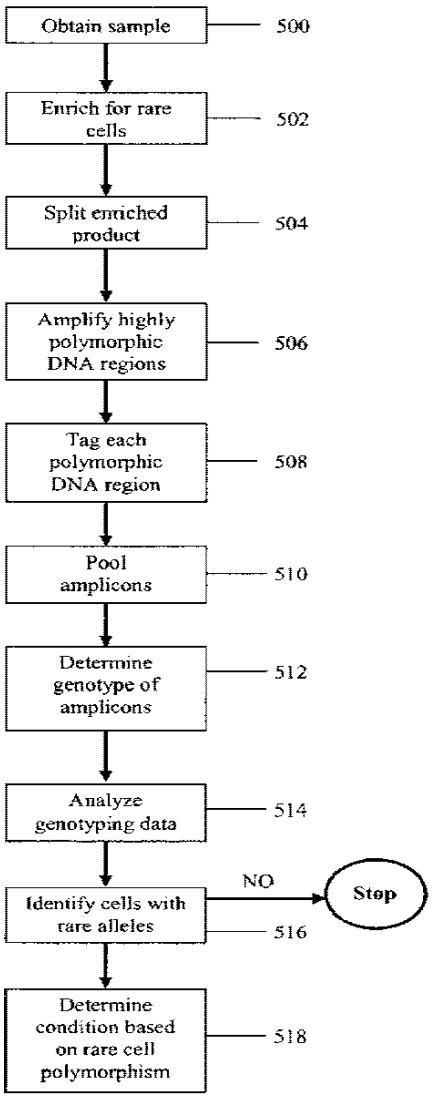

Obtain sample
500
Enrich for rare cells
502
Split enriched product
504
Amplify highly polymorphic DNA regions
506
Tag each polymorphic DNA region
508
Pool amplicons
510
Determine genotype of amplicons
512
Analyze genotyping data
514
Identify cells with rare alleles
NO
Stop
516
Determine condition based on rare cell polymorphism
518

Figure 5

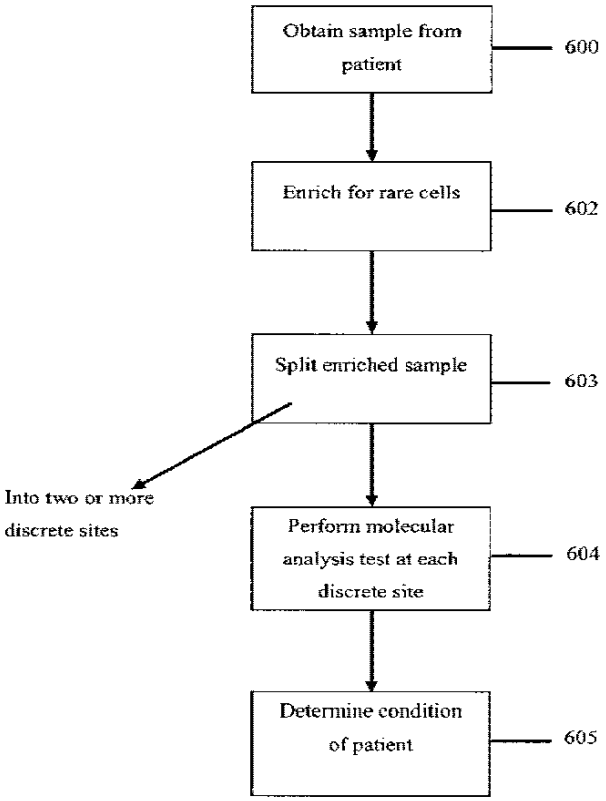
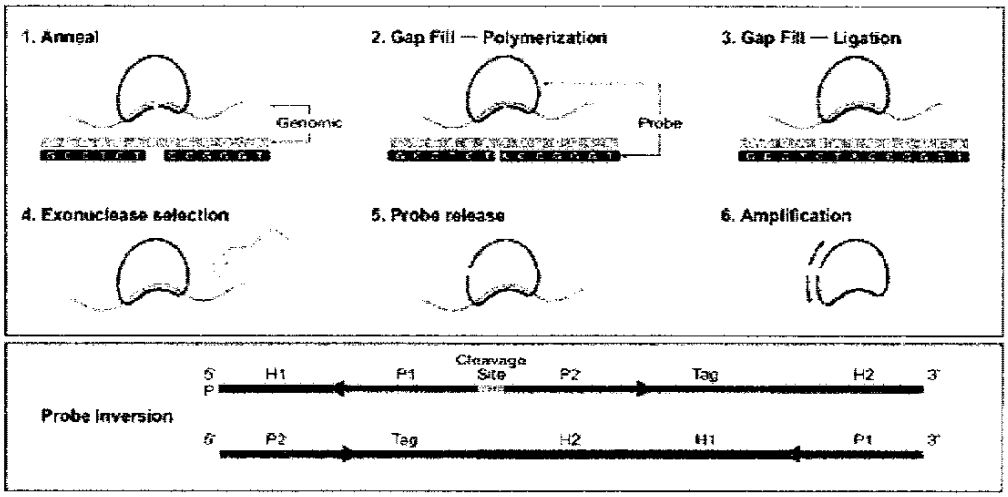

Obtain sample from patient
600
Enrich for rare cells
602
Split enriched sample
603
Into two or more discrete sites
Perform molecular analysis test at each discrete site
604
Determine condition of patient
605

Figure 6



Molecular Inversion Probes are so named because the oligonucleotide probe central to the process undergoes a unimolecular rearrangement from a molecule that cannot be amplified (step 1), into a molecule that can be amplified (step 6). This rearrangement is mediated by hybridization to genomic DNA (step 2) and an enzymatic "gap fill" process that occurs in an allele-specific manner (step 3). The resulting circularized probe can be separated from cross-reacted or unreacted probes by a simple exonuclease reaction (step 4). Figure 1 shows these steps.

Figure 7A

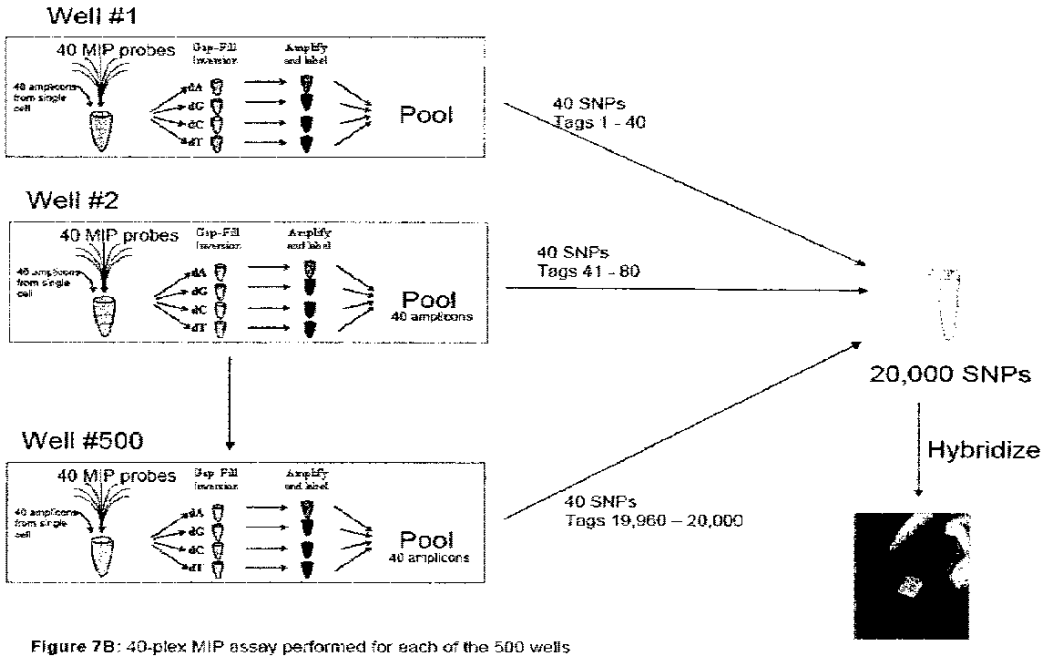
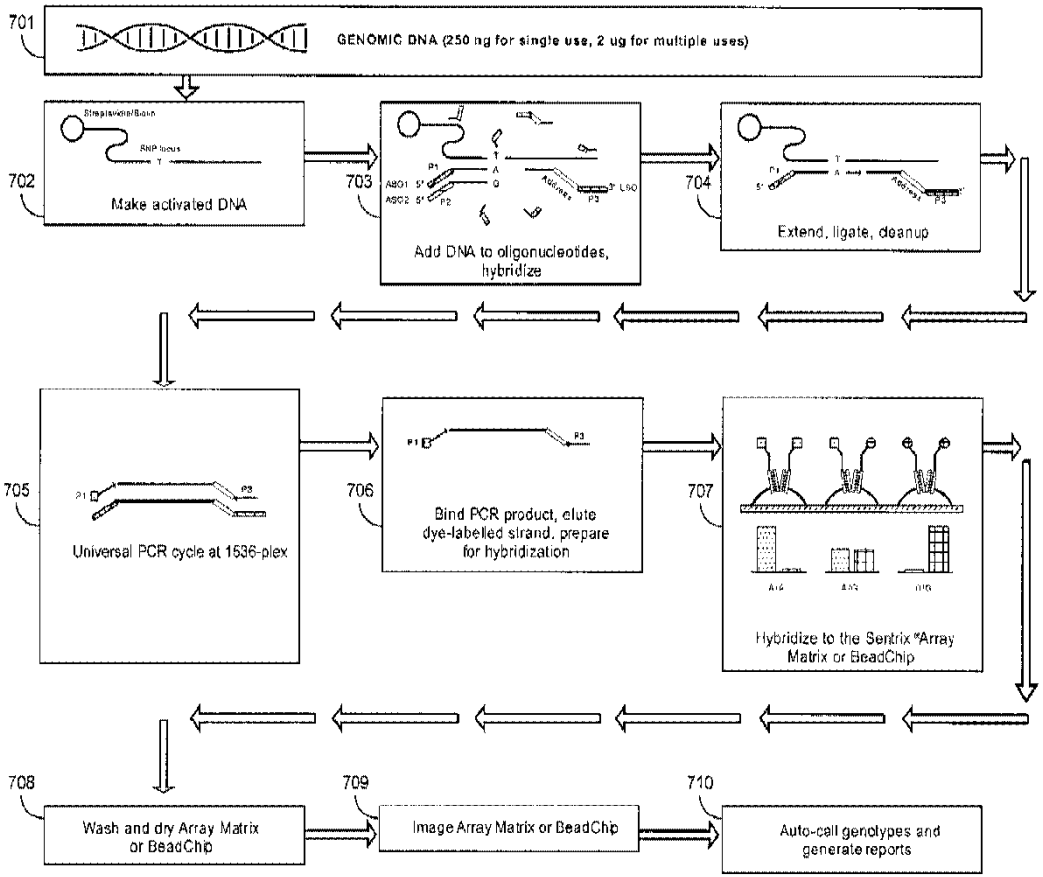


Figure 7B: 40-plex MIP assay performed for each of the 500 wells

Figure 7C


701
GENOMIC DNA (250 ng for single use, 2 ug for multiple uses)
702
Make activated DNA
703
Add DNA to oligonucleotides, hybridize
704
Extend, ligate, deanup
705
Universal PCR cycle at 1536-plex
706
Bind PCR product, elute dye-labelled strand, prepare for hybridization
707
Hybridize to the Sentrix *Array Matrix or BeadChip
708
Wash and dry Array Matrix or BeadChip
709
Image Array Matrix or BeadChip
710
Auto-call genotypes and generate reports

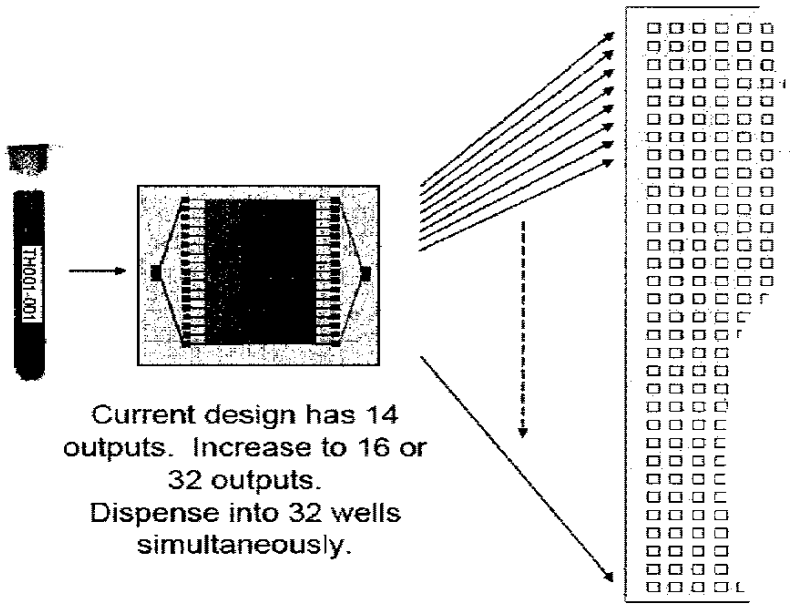
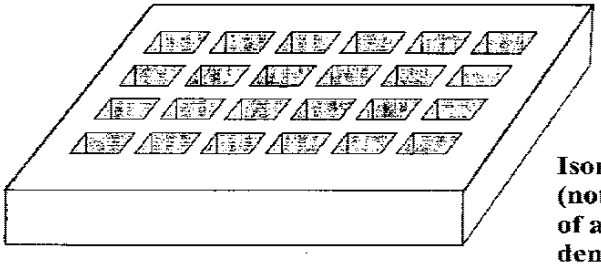

Current design has 14 outputs. Increase to 16 or 32 outputs.
Dispense into 32 wells simultaneously.
1536 well plate
32 x 48 wells

Figure 8A

Figure 8B

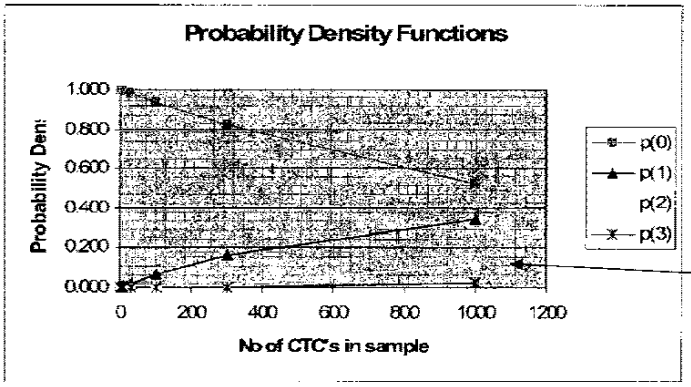


Isometric View (not illustrative of actual well density)

Figure 8C



Cross-section



Probability of two CTCs in same well

Figure 9

Figure 10

2AR
A DISINTEGRIN
ACTIVATOR OF THYROID AND RETINOIC ACID RECEPTOR (ACTR)
ADAM 11
ADIPOGENESIS INHIBITORY FACTOR (ADIF)
ALPHA 6 INTEGRIN SUBUNIT
ALPHA V INTEGRIN SUBUNIT
ALPHA-CATENIN
AMPLIFIED IN BREAST CANCER 1 (AIB1)
AMPLIFIED IN BREAST CANCER 3 (AIB3)
AMPLIFIED IN BREAST CANCER 4 (AIB4)
AMYLOID PRECURSOR PROTEIN SECRETASE (APPS)
AP-2 GAMMA
APPS
ATP-BINDING CASSETTE TRANSPORTER (ABCT)
PLACENTA-SPECIFIC (ABCP)
ATP-BINDING CASSETTE SUBFAMILY C MEMBER (ABCC1)
BAG-1
BASIGIN (BSG)
BCEI
B-CELL DIFFERENTIATION FACTOR (BCDF)
B-CELL LEUKEMIA 2 (BCL-2)
B-CELL STIMULATORY FACTOR-2 (BSF-2)
BCL-1
BCL-2-ASSOCIATED X PROTEIN (BAX)
BCRP
BETA 1 INTEGRIN SUBUNIT
BETA 3 INTEGRIN SUBUNIT
BETA 5 INTEGRIN SUBUNIT
BETA-2 INTERFERON
BETA-CATENIN
BETA-CATENIN
BONE SIALOPROTEIN (BSP)
BREAST CANCER ESTROGEN-INDUCIBLE SEQUENCE (BCEI)
BREAST CANCER RESISTANCE PROTEIN (BCRP)
BREAST CANCER TYPE 1 (BRCA1)
BREAST CANCER TYPE 2 (BRCA2)
BREAST CARCINOMA AMPLIFIED SEQUENCE 2 (BCAS2)
CADHERIN
EPITHELIAL CADHERIN-11
CADHERIN-ASSOCIATED PROTEIN
CALCITONIN RECEPTOR (CTR)
CALCIUM PLACENTAL PROTEIN (CAPL)
CALCYCLIN
CALLA
CAM5
CAPL
CARCINOEMBRYONIC ANTIGEN (CEA)
CATENIN
ALPHA 1
CATHEPSIN B
CATHEPSIN D
CATHEPSIN K
CATHEPSIN L2
CATHEPSIN O
CATHEPSIN O1
CATHEPSIN V
CD10
CD146
CD147
CD24
CD29
CD44
CD51
CD54
CD61
CD66e
CD82
CD87
CD9
CEA
CELLULAR RETINOL-BINDING PROTEIN 1 (CRBP1)
c-ERBB-2
CK7
CK8
CK18
CK19
CK20
CLAUDIN-7
c-MET
COLLAGENASE
FIBROBLAST
COLLAGENASE
INTERSTITIAL
COLLAGENASE-3
COMMON ACUTE LYMPHOCYTIC LEUKEMIA ANTIGEN (CALLA)
CONNEXIN 26 (Cx26)
CONNEXIN 43 (Cx43)
CORTACTIN
COX-2
CTLA-8
CTR
CTSD
CYCLIN D1
CYCLOOXYGENASE-2
CYTOKERATIN 18
CYTOKERATIN 19
CYTOKERATIN 8
CYTOTOXIC T-LYMPHOCYTE-ASSOCIATED SERINE ESTERASE 8 (CTLA-8)

DIFFERENTIATION-INHIBITING ACTIVITY (DIA)
DNA AMPLIFIED IN MAMMARY CARCINOMA 1 (DAM1)
DNA TOPOISOMERASE II ALPHA
DR-NM23
E-CADHERIN
EMMPRIN
EMS1
ENDOTHELIAL CELL GROWTH FACTOR (ECGF)
PLATELET-DERIVED (PD-ECGF)
ENKEPHALINASE
EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)
EPISIALIN
EPITHELIAL MEMBRANE ANTIGEN (EMA)
ER-ALPHA
ERBB2
ERBB4
ER-BETA
ERF-1
ERYTHROID-POTENTIATING ACTIVITY (EPA)
ESR1
ESTROGEN RECEPTOR-ALPHA
ESTROGEN RECEPTOR-BETA
ETS-1
EXTRACELLULAR MATRIX METALLOPROTEINASE INDUCER (EMMPRIN)
FIBRONECTIN RECEPTOR
BETA POLYPEPTIDE (FNRB)
FIBRONECTIN RECEPTOR BETA SUBUNIT (FNRB)
FLK-1
GA15.3
GA733.2
GALECTIN-3
GAMMA-CATENIN
GAP JUNCTION PROTEIN (26 kDa)
GAP JUNCTION PROTEIN (43 kDa)
GAP JUNCTION PROTEIN ALPHA-1 (GJA1)
GAP JUNCTION PROTEIN BETA-2 (GJB2)
GCP1
GELATINASE A
GELATINASE B
GELATINASE (72 kDa)
GELATINASE (92 kDa)
GLIOSTATIN
GLUCOCORTICOID RECEPTOR INTERACTING PROTEIN 1 (GRIP1)
GLUTATHIONE S-TRANSFERASE p
GM-CSF
GRANULOCYTE CHEMOTACTIC PROTEIN 1 (GCP1)
GRANULOCYTE-MACROPHAGE-COLONY STIMULATING FACTOR
GROWTH FACTOR RECEPTOR BOUND-7 (GRB-7)
GSTp
HAP
HEAT-SHOCK COGNATE PROTEIN 70 (HSC70)
HEAT-STABLE ANTIGEN
HEPATOCYTE GROWTH FACTOR (HGF)
HEPATOCYTE GROWTH FACTOR RECEPTOR (HGFR)
HEPATOCYTE-STIMULATING FACTOR III (HSF III)
HER-2
HER2/NEU
HERMES ANTIGEN
HET
HHM
HUMORAL HYPERCALCEMIA OF MALIGNANCY (HHM)
ICERE-1
INT-1
INTERCELLULAR ADHESION MOLECULE-1 (ICAM-1)
INTERFERON-GAMMA-INDUCING FACTOR (IGIF)
INTERLEUKIN-1 ALPHA (IL-1A)
INTERLEUKIN-1 BETA (IL-1B)
INTERLEUKIN-11 (IL-11)
INTERLEUKIN-17 (IL-17)
INTERLEUKIN-18 (IL-18)
INTERLEUKIN-6 (IL-6)
INTERLEUKIN-8 (IL-8)
INVERSELY CORRELATED WITH ESTROGEN RECEPTOR EXPRESSION-1 (ICERE-1)
KAI1
KDR
KERATIN 8
KERATIN 18
KERATIN 19
KISS-1
LEUKEMIA INHIBITORY FACTOR (LIF)
LIF
LOST IN INFLAMMATORY BREAST CANCER (LIBC)
LOT ("LOST ON TRANSFORMATION")
LYMPHOCYTE HOMING RECEPTOR
MACROPHAGE-COLONY STIMULATING FACTOR
MAGE-3
MAMMAGLOBIN
MASPIN
MC56
M-CSF
MDC
MDNCF
MDR
MELANOMA CELL ADHESION MOLECULE (MCAM)
MEMBRANE METALLOENDOPEPTIDASE (MME)

MEMBRANE-ASSOCIATED NEUTRAL ENDOPEPTIDASE (NEP)
CYSTEINE-RICH PROTEIN (MDC)
METASTASIN (MTS-1)
MLN64
MMP1
MMP2
MMP3
MMP7
MMP9
MMP11
MMP13
MMP14
MMP15
MMP16
MMP17
MOESIN
MONOCYTE ARGININE-SERPIN
MONOCYTE-DERIVED NEUTROPHIL CHEMOTACTIC FACTOR
MONOCYTE-DERIVED PLASMINOGEN ACTIVATOR INHIBITOR
MTS-1
MUC-1
MUC18
MUCIN LIKE CANCER ASSOCIATED ANTIGEN (MCA)
MUCIN
MUC-1
MULTIDRUG RESISTANCE PROTEIN 1 (MDR, MDR1)
MULTIDRUG RESISTANCE RELATED PROTEIN-1 (MRP, MRP-1)
N-CADHERIN
NEP
NEU
NEUTRAL ENDOPEPTIDASE
NEUTROPHIL-ACTIVATING PEPTIDE 1 (NAP1)
NM23-H1
NM23-H2
NME1
NME2
NUCLEAR RECEPTOR COACTIVATOR-1 (NCoA-1)
NUCLEAR RECEPTOR COACTIVATOR-2 (NCoA-2)
NUCLEAR RECEPTOR COACTIVATOR-3 (NCoA-3)
NUCLEOSIDE DIPHOSPHATE KINASE A (NDPKA)
NUCLEOSIDE DIPHOSPHATE KINASE B (NDPKB)
ONCOSTATIN M (OSM)
ORNITHINE DECARBOXYLASE (ODC)
OSTEOCLAST DIFFERENTIATION FACTOR (ODF)
OSTEOCLAST DIFFERENTIATION FACTOR RECEPTOR (ODFR)
OSTEONECTIN (OSN, ON)
OSTEOPONTIN (OPN)
OXYTOCIN RECEPTOR (OXTR)
p27/kip1
p300/CBP COINTEGRATOR ASSOCIATE PROTEIN (p/CIP)
p53
p9Ka
PAI-1
PAI-2
PARATHYROID ADENOMATOSIS 1 (PRAD1)
PARATHYROID HORMONE-LIKE HORMONE (PTHLH)
PARATHYROID HORMONE-RELATED PEPTIDE (PTHrP)
P-CADHERIN
PD-ECGF
PDGF-1
PEANUT-REACTIVE URINARY MUCIN (PUM)
P-GLYCOPROTEIN (P-GP)
PGP-1
PHGS-2
PHS-2
PIP
PLAKOGLOBIN
PLASMINOGEN ACTIVATOR INHIBITOR (TYPE 1)
PLASMINOGEN ACTIVATOR INHIBITOR (TYPE 2)
PLASMINOGEN ACTIVATOR (TISSUE-TYPE)
PLASMINOGEN ACTIVATOR (UROKINASE-TYPE)
PLATELET GLYCOPROTEIN IIIa (GP3A)
PLAU
PLEOMORPHIC ADENOMA GENE-LIKE 1 (PLAGL1)
POLYMORPHIC EPITHELIAL MUCIN (PEM)
PRAD1
PROGESTERONE RECEPTOR (PgR)
PROGESTERONE RESISTANCE
PROSTAGLANDIN ENDOPEROXIDE SYNTHASE-2
PROSTAGLANDIN G/H SYNTHASE-2
PROSTAGLANDIN H SYNTHASE-2
pS2
PS6K
PSORIASIN
PTHLH
PTHrP
RAD51
RAD52
RAD54
RAP46
RECEPTOR-ASSOCIATED COACTIVATOR 3 (RAC3)
REPRESSOR OF ESTROGEN RECEPTOR ACTIVITY (REA)
S100A4

S100A6
S100A7
S6K
SART-1
SCAFFOLD ATTACHMENT FACTOR B (SAF-B)
SCATTER FACTOR (SF)
SECRETED PHOSPHOPROTEIN-1 (SPP-1)
SECRETED PROTEIN ACIDIC AND RICH IN CYSTEINE (SPARC)
STANNICALCIN
STEROID RECEPTOR COACTIVATOR-1 (SRC-1)
STEROID RECEPTOR COACTIVATOR-2 (SRC-2)
STEROID RECEPTOR COACTIVATOR-3 (SRC-3)
STEROID RECEPTOR RNA ACTIVATOR (SRA)
STROMELYSIN-1
STROMELYSIN-3
TENASCIN-C (TN-C)
TESTES-SPECIFIC PROTEASE 50
THROMBOSPONDIN I
THROMBOSPONDIN II
THYMIDINE PHOSPHORYLASE (TP)
THYROID HORMONE RECEPTOR ACTIVATOR MOLECULE 1 (TRAM-1)
TIGHT JUNCTION PROTEIN 1 (TJP1)
TIMP1
TIMP2
TIMP3
TIMP4
TISSUE-TYPE PLASMINOGEN ACTIVATOR
TN-C
TP53
tPA
TRANSCRIPTIONAL INTERMEDIARY FACTOR 2 (TIF2)
TREFOIL FACTOR 1 (TFF1)
TSG101
TSP-1
TSP1
TSP-2
TSP2
TSP50
TUMOR CELL COLLAGENASE STIMULATING FACTOR (TCSF)
TUMOR-ASSOCIATED EPITHELIAL MUCIN
uPA
uPAR
UROKINASE
UROKINASE-TYPE PLASMINOGEN ACTIVATOR
UROKINASE-TYPE PLASMINOGEN ACTIVATOR RECEPTOR (uPAR)
UVOMORULIN
VASCULAR ENDOTHELIAL GROWTH FACTOR
VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR-2 (VEGFR2)
VASCULAR ENDOTHELIAL GROWTH FACTOR-A
VASCULAR PERMEABILITY FACTOR
VEGFR2
VERY LATE T-CELL ANTIGEN BETA (VLA-BETA)
VIMENTIN
VITRONECTIN RECEPTOR ALPHA POLYPEPTIDE (VNRA)
VITRONECTIN RECEPTOR
VON WILLEBRAND FACTOR
VPF
VWF
WNT-1
ZAC
ZO-1
ZONULA OCCLUDENS-1

Name	SEQ ID NO	Sequence (5' to 3')	Exon	Amplicon Size
NXK-ex18.1(+)	11	TCAGAGCCTGTGTTTCTACCAA	18	534
NXK-ex18.2(-)	12	TGGTCTCACAGGACCACTGATT	18	
NXK-ex18.3(+)	13	TCCAAATGAGCTGGCAAGTG	18	397
NXK-ex18.4(-)	14	TCCCAAACACTCAGTGAAACAAA	18	
NXK-ex19.1(+)	15	AAATAATCAGTGTGATTCGTGGAG	19	495
NXK-ex19.2(-)	16	GAGGCCAGTGCTGTCTCTAAGG	19	
NXK-ex19.3(+)	17	GTGCATCGCTGGTAACATCC	19	298
NXK-ex19.4(-)	18	TGTGGAGATGAGCAGGGTCT	19	
NXK-ex20.1(+)	19	ACTTCACAGCCCTGCGTAAAC	20	555
NXK-ex20.2(-)	20	ATGGGACAGGCACTGATTTGT	20	
NXK-ex20.3(+)	21	ATCGCATTCATGCGTCTTCA	20	379
NXK-ex20.4(-)	22	ATCCCCATGGCAAACTCTTG	20	
NXK-ex21.1(+)	23	GCAGCGGGTTACATCTTCTTTC	21	526
NXK-ex21.2(-)	24	CAGCTCTGGCTCACACTACCAG	21	
NXK-ex21.3(+)	25	GCAGCGGGTTACATCTTCTTTC	21	349
NXK-ex21.4(-)	26	CATCCTCCCCTGCATGTGT	21	

Figure 11

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 00937

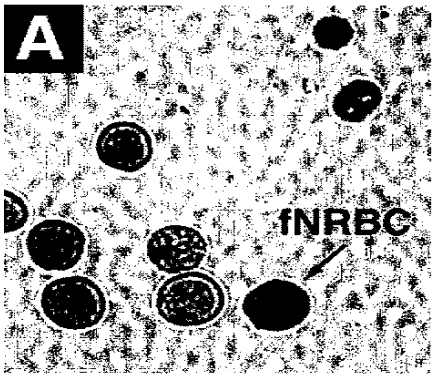


Figure 12 A

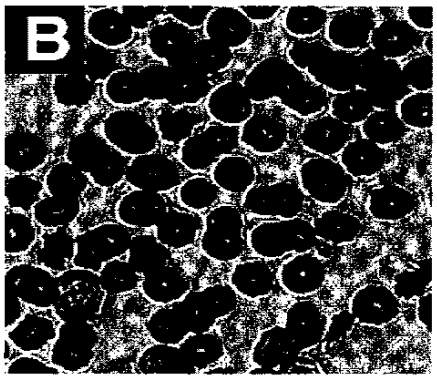


Figure 12 B

10x Genomics, Inc. Exhibit 2004
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IPR2023-00955
Page 00938

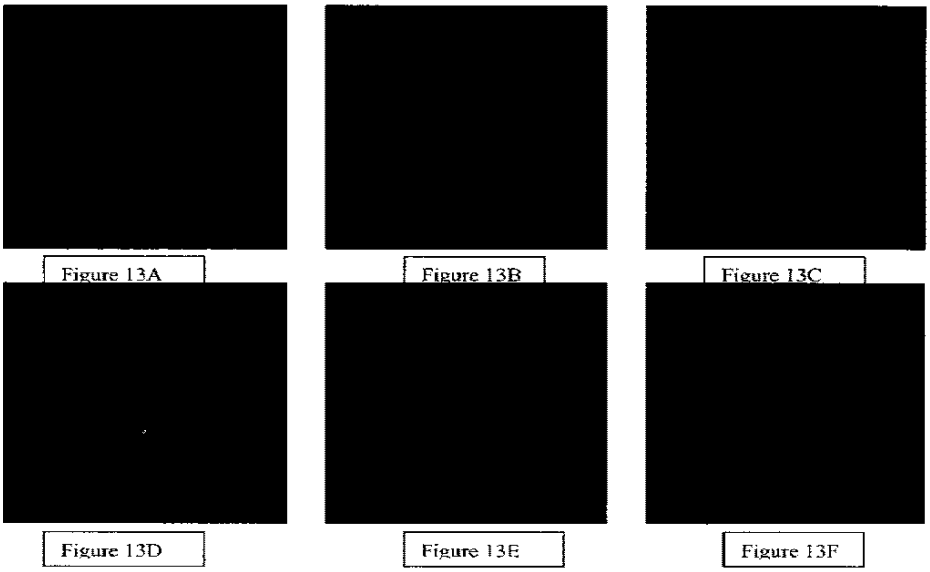


Figure 13A-F (Blue= nucleus, Red = X chromosome, Green = Y chromosome)

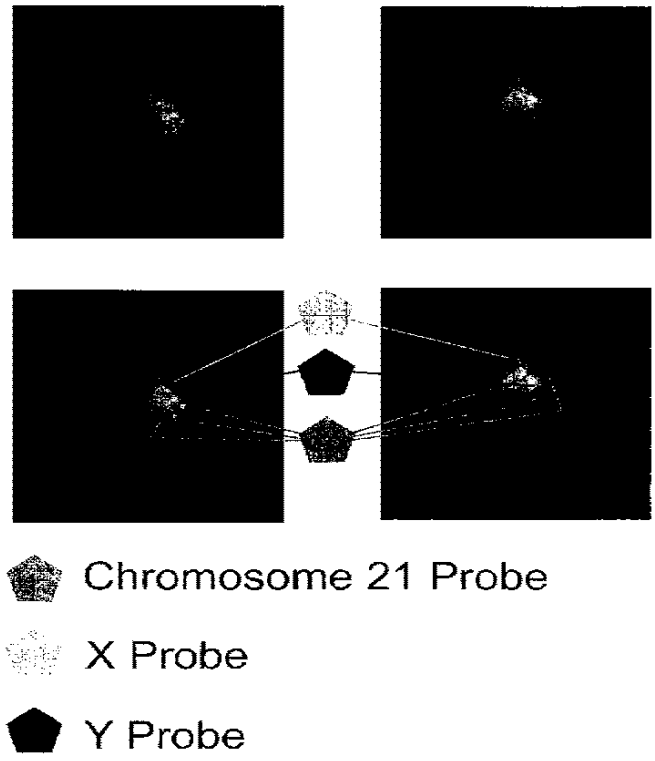


Figure 14

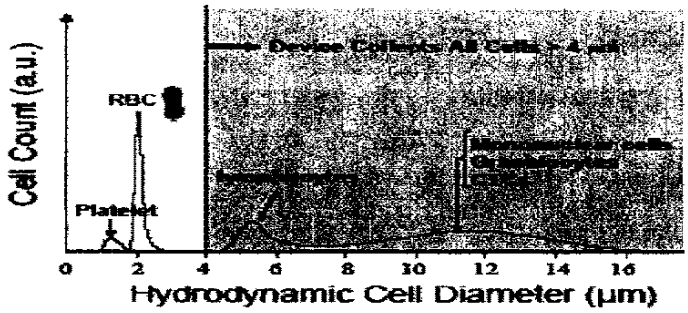

Cell Count (a.u.)
RBC
Platelet
0
2
4
6
8
10
12
14
16
Hydrodynamic Cell Diameter (µm)

Figure 15

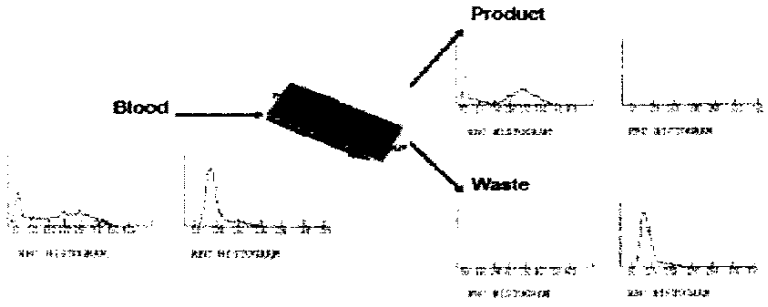


Figure 16

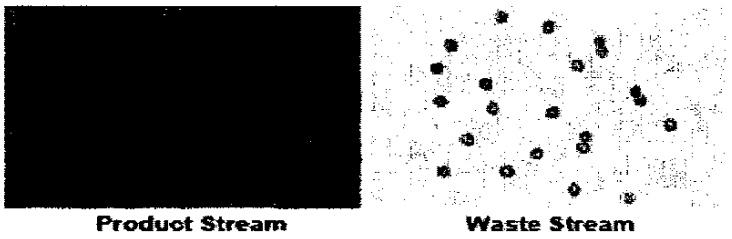


Figure 17

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Figure 18

10x Genomics, Inc. Exhibit 2004
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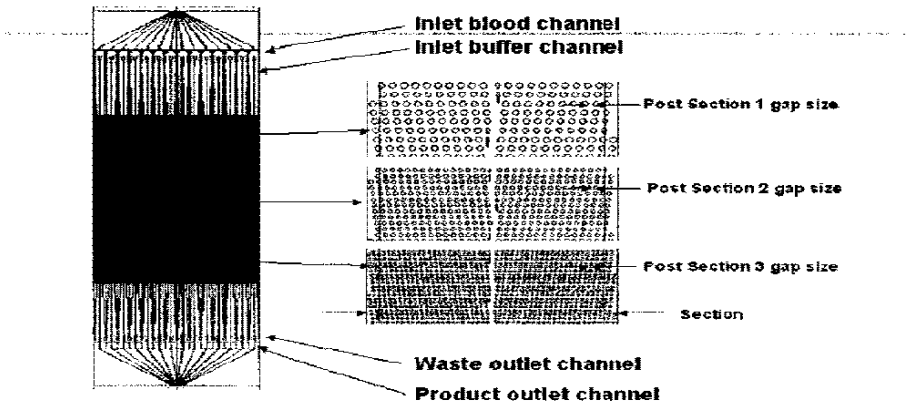
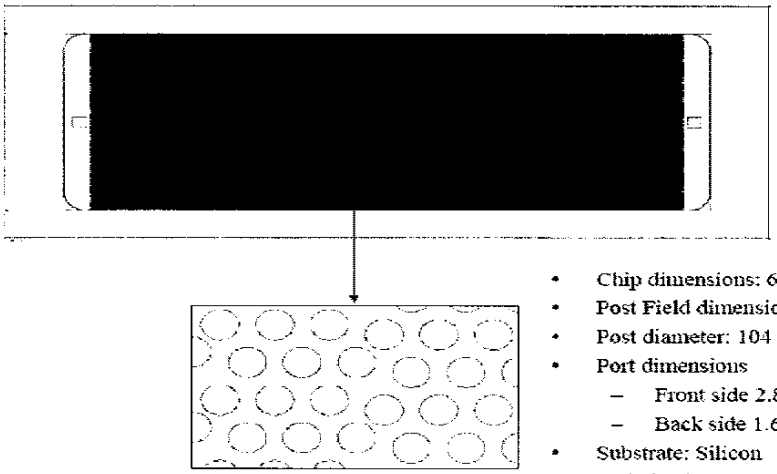

Inlet blood channel
Inlet buffer channel
Post Section 1 gap size
Post Section 2 gap size
Post Section 3 gap size
Section
Waste outlet channel
Product outlet channel

Figure 19A

		Version 1	Version 2	Version 3
Inlet channel width (blood)	50	50	100	100
Inlet channel width (buffer)	55	55	110	110
Outlet channel width (product)	49	49	98	98
Outlet channel width (waste)	50	50	100	100
Gap size / Deflect cell size				
Post section 1	18/9	36/18	44/22	50/25
Post section 2	12/6	24/12	30/15	36/18
Post section 3	8/4	16/8	20/10	24/12
Number of parallel sections	14	14	14	14
Etch depth	150	150	150	150
Product cell size (cut off)	4	8	10	12
Estimated Flow rate, ml/u	5	10	20	30

Figure 19B



- Chip dimensions: 66.0 x 24.9 mm
- Post Field dimensions: 51.3 x 18.9 mm
- Post diameter: 104 μm
- Port dimensions
  - Front side 2.83 x2.83 mm
  - Back side 1.66 x 1.66 mm
- Substrate: Silicon
- Etch depth: 100 μm

Figure 19C

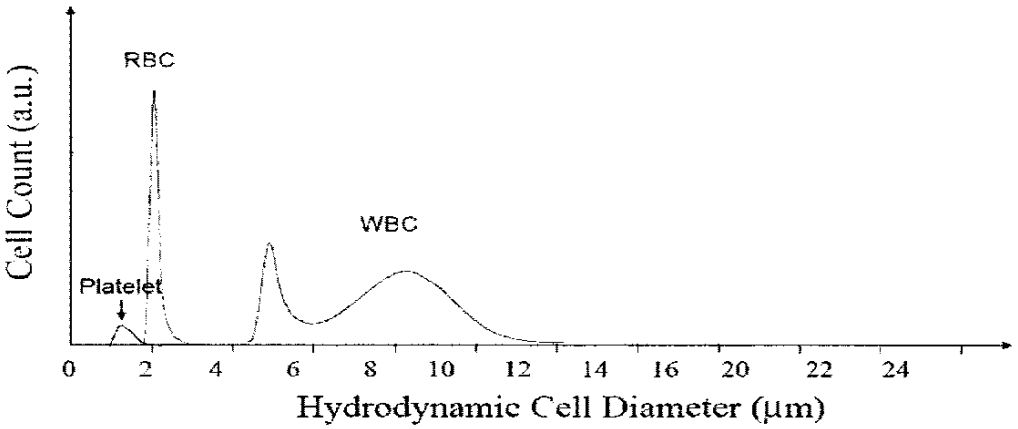


Figure 20A

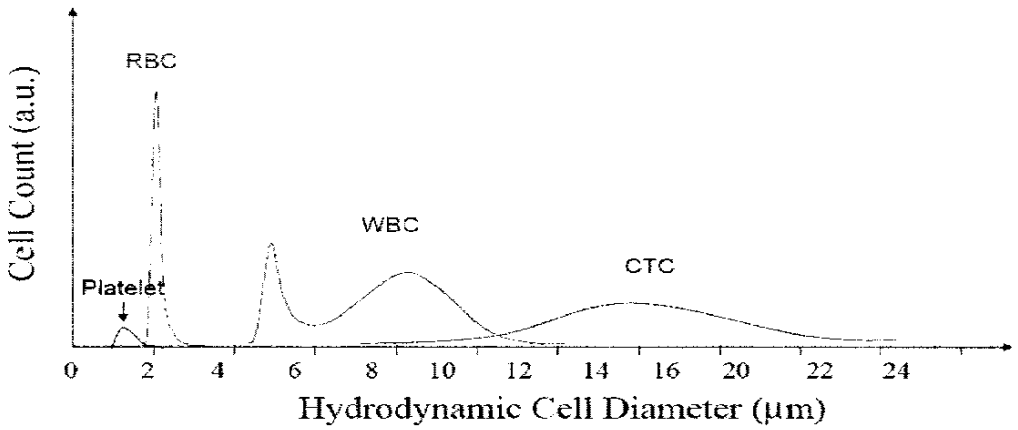


Figure 20B

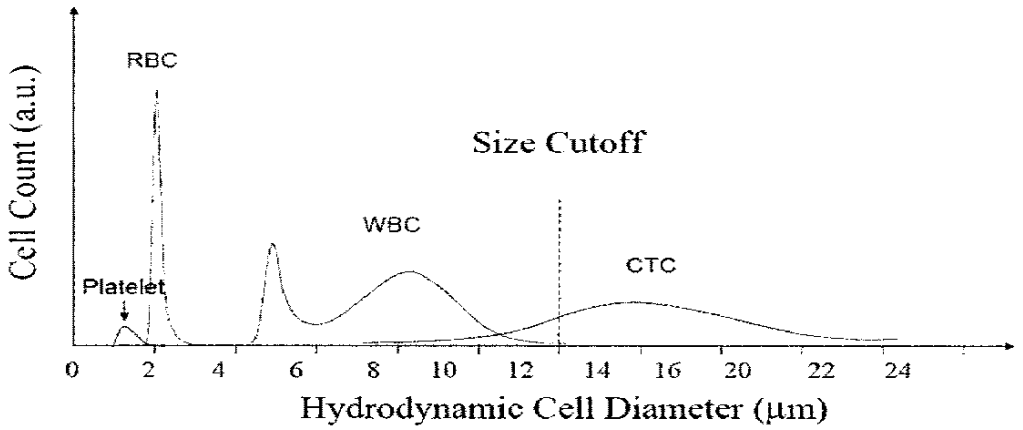


Figure 20C

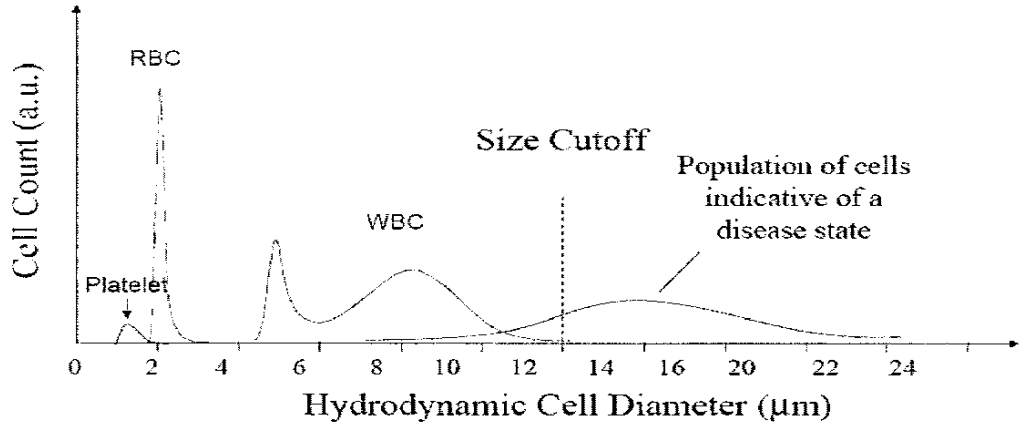


Figure 20D

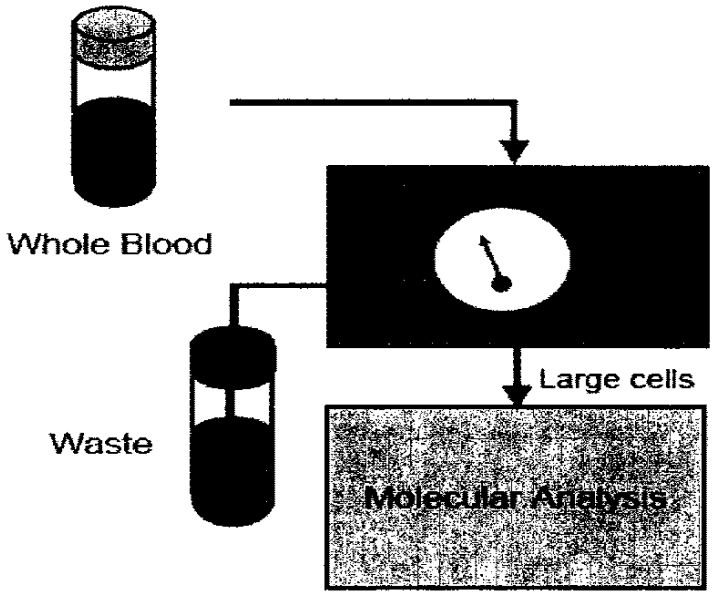


Figure 21

10x Genomics, Inc. Exhibit 2004
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Page 00950

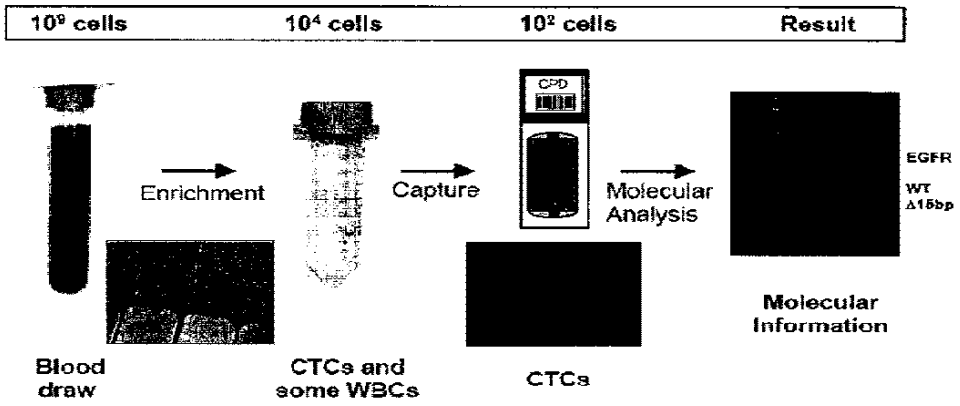


Figure 22

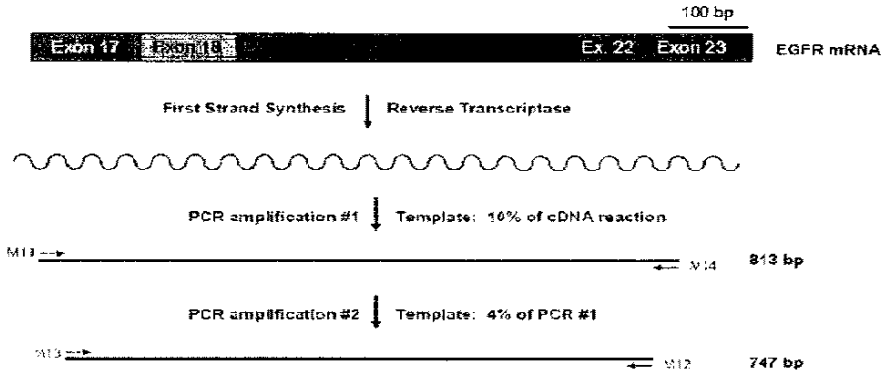


Figure 23

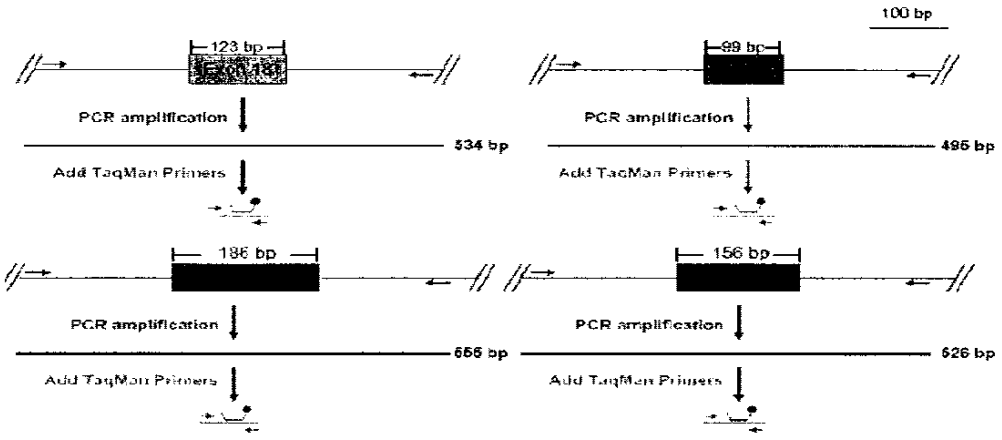

100 bp
123 bp
99 bp
PCR amplification
534 bp
PCR amplification
495 bp
Add TaqMan Primers
Add TaqMan Primers
186 bp
156 bp
PCR amplification
556 bp
PCR amplification
526 bp
Add TaqMan Primers
Add TaqMan Primers

Figure 24

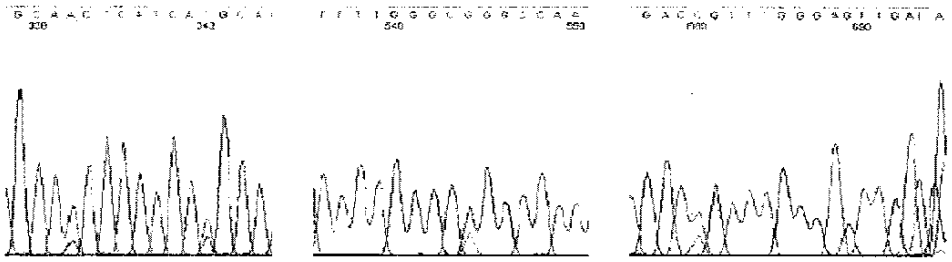
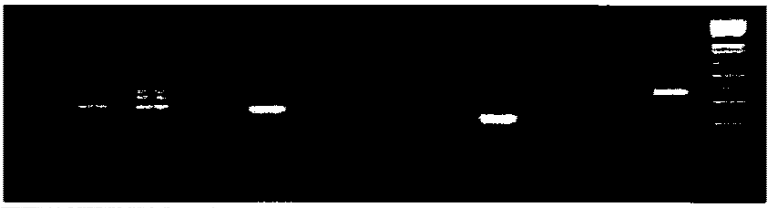

CAA → CAG (silent) at 2361
ACG → ATG (T790M) at 2369
CTG → CGG (L828R) at 2573
ACC → ACT (silent) at 2709

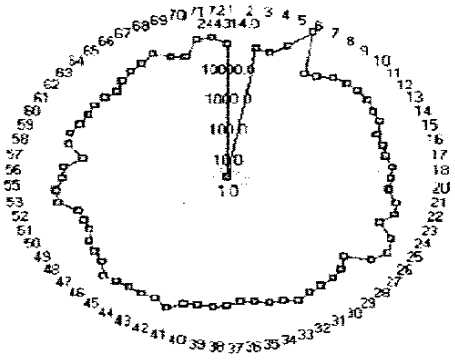
Figure 25



WBC cDNA	-	+	-	-	+	-	-	+	-	-	+	-
H1650 cDNA	-	-	+	-	-	+	-	-	+	-	-	+
Target mRNA	BCKD			CD45			EpCaM			EGFR		

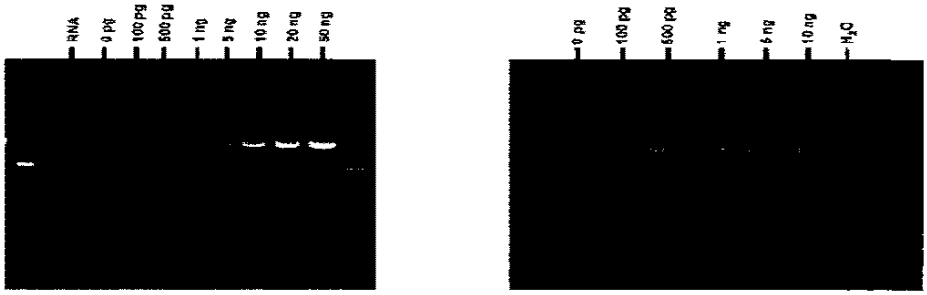
Sizer Standards

Figure 26A



	Tissue	Ct	[mRNA]
1	Renal Blood Vessel	24.02	25,916
2	Blood Mononuclear Cells (PBMC)	>40	0
3	Colon	24.28	21,968
4	Primary Lung Bronchus	24.49	19,224

Figure 26B



PCR #1 PCR #2 (Nested)

NIH-3T3 – 100 pg total RNA = ~20 cells
NCI-H1975 -- 100 pg total RNA = ~10 cells
COS-7 – 100 pg total RNA = ~6 cells

Figure 27

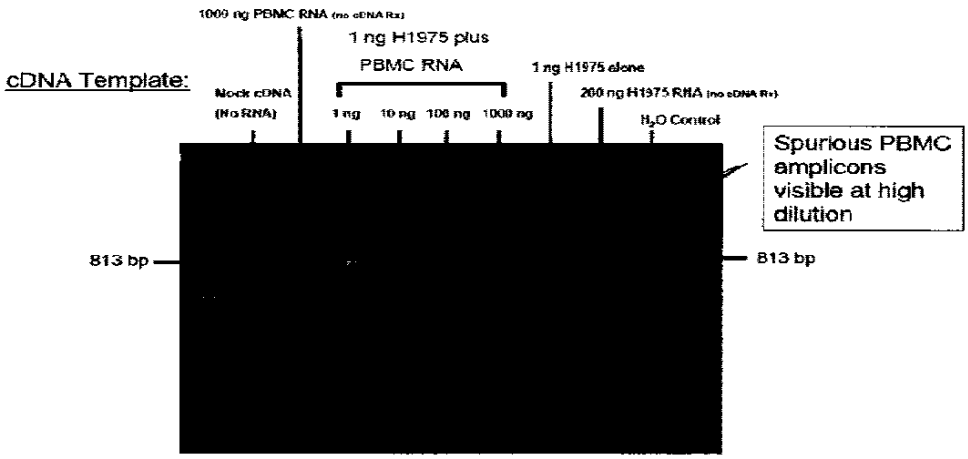


Figure 28A

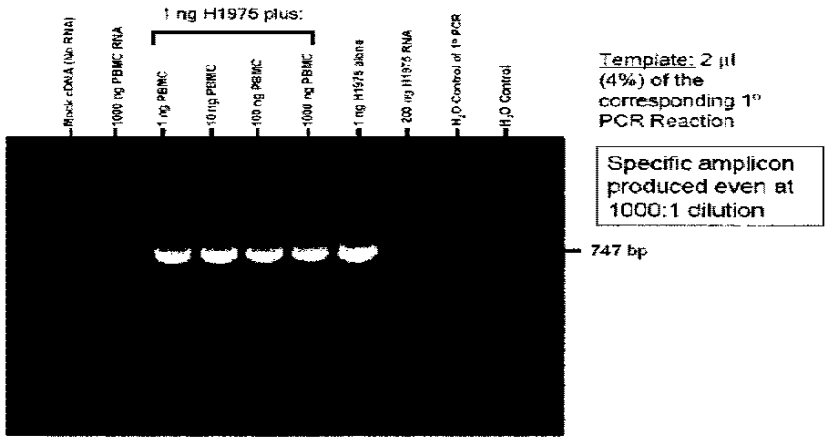


Figure 28B

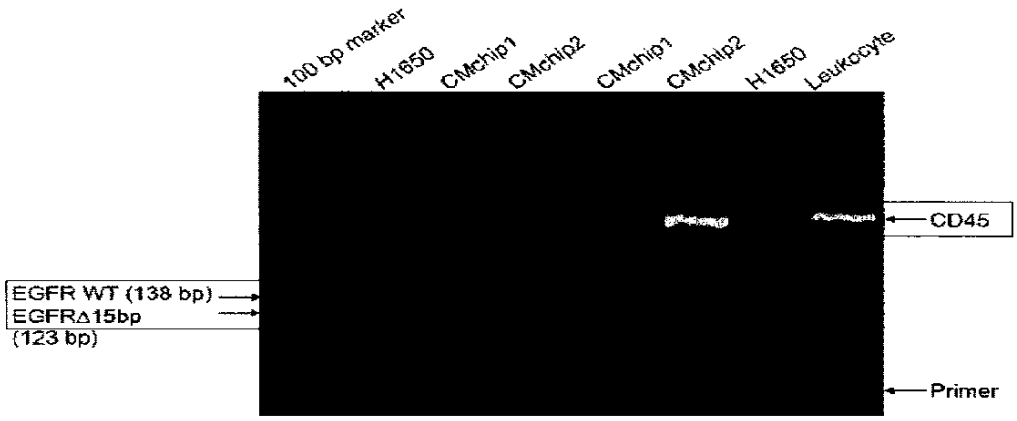

100 bp marker
H1650
CMchip1
CMchip2
CMchip1
CMchip2
H1650
Leukocyte
CD45
EGFR WT (138 bp)
EGFRΔ15bp
(123 bp)
Primer

Figure 29

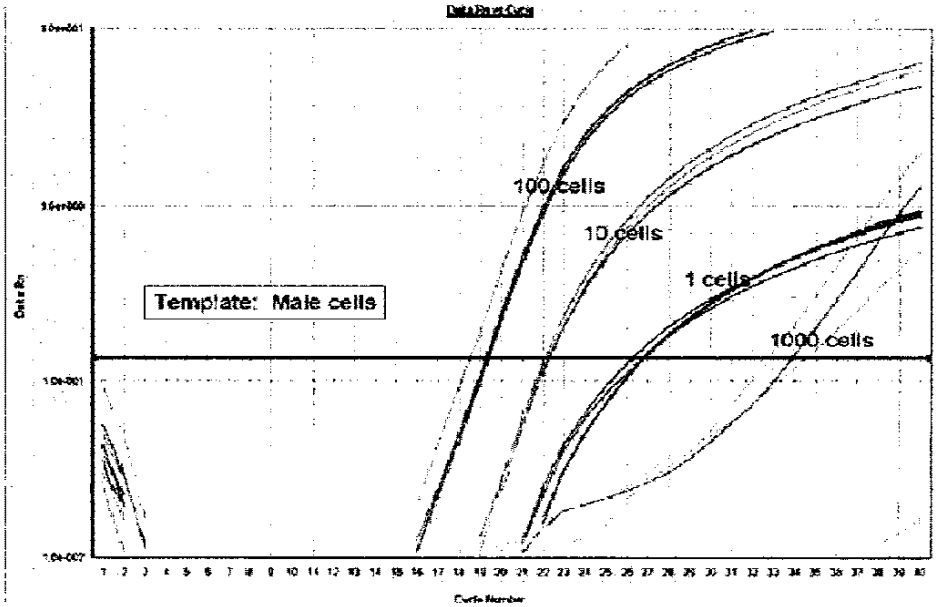


FIGURE 30

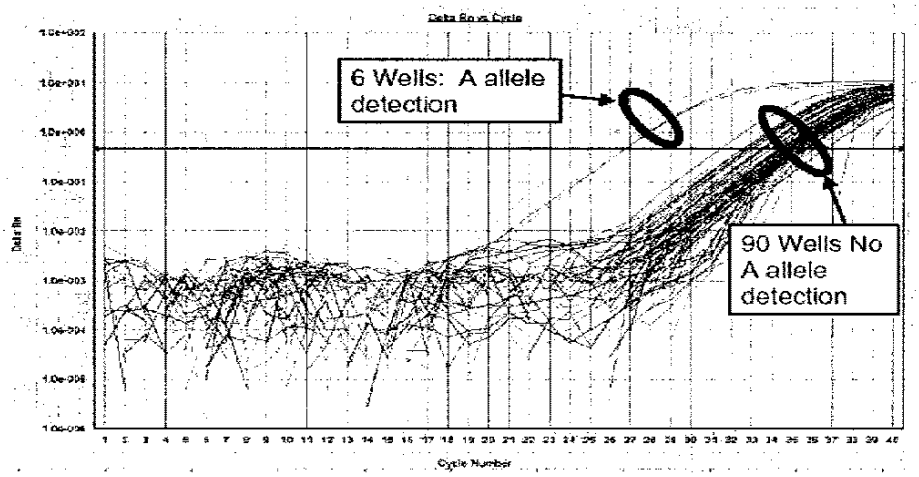

6 Wells: A allele detection
90 Wells No A allele detection
Cycle Number

FIGURE 31

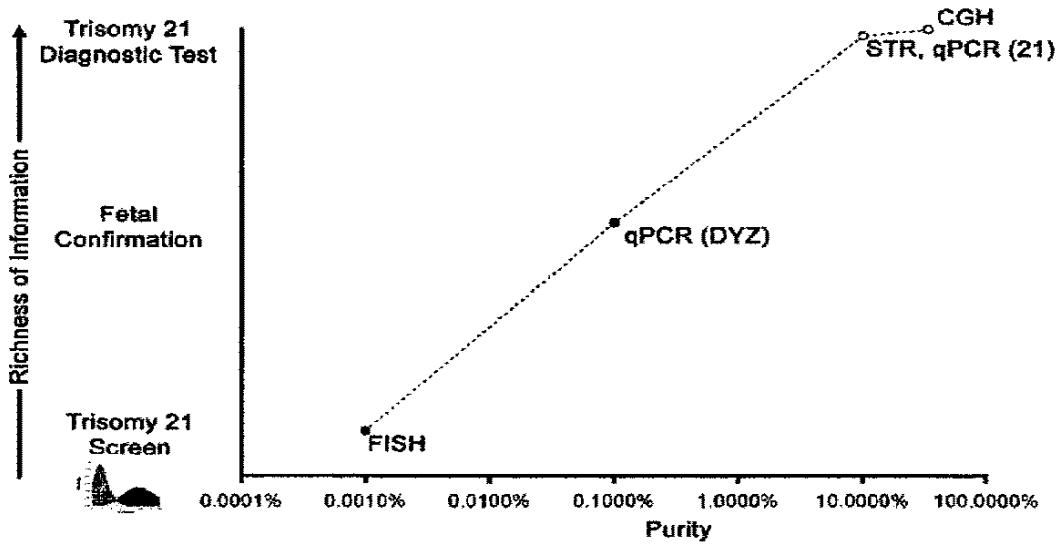

Trisomy 21
Diagnostic Test
Fetal
Confirmation
Trisomy 21
Screen
Richness of Information
CGH
STR, qPCR (21)
qPCR (DYZ)
FISH
0.0001%
0.0010%
0.0100%
0.1000%
1.0000%
10.0000%
100.0000%
Purity

FIGURE 32

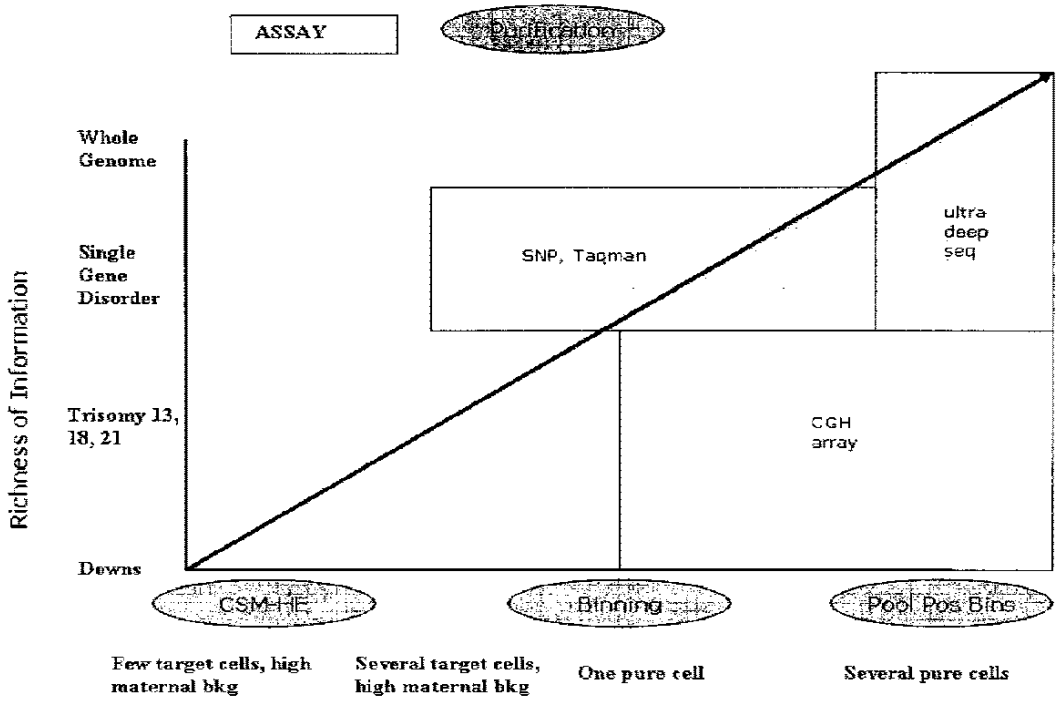

ASSAY
Purification
Whole Genome
Single Gene Disorder
Trisomy 13, 18, 21
Downs
Richness of Information
SNP, Taqman
ultra deep seq
CGH array
CSM-HE
Binning
Pool Pos Bins
Few target cells, high maternal bkg
Several target cells, high maternal bkg
One pure cell
Several pure cells

FIGURE 33

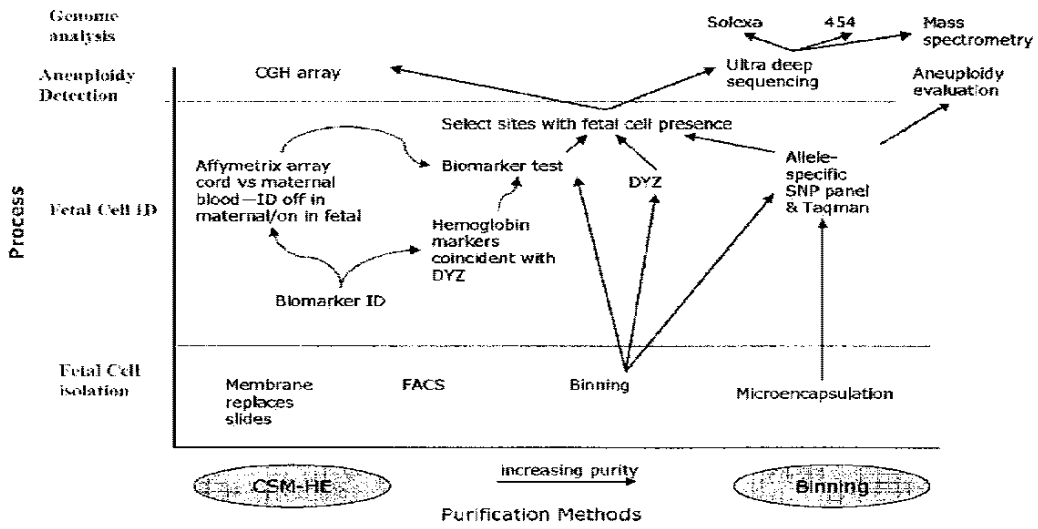

Genomic analysis
Solexa
454
Mass spectrometry
Aneuploidy Detection
CGH array
Ultra deep sequencing
Aneuploidy evaluation
Select sites with fetal cell presence
Affymetrix array cord vs maternal blood—ID off in maternal/on in fetal
Biomarker test
DYZ
Allele-specific SNP panel & Taqman
Process
Fetal Cell ID
Hemoglobin markers coincident with DYZ
Biomarker ID
Fetal Cell isolation
Membrane replaces slides
FACS
Binning
Microencapsulation
CSM-HE
increasing purity
Binning
Purification Methods

FIGURE 34

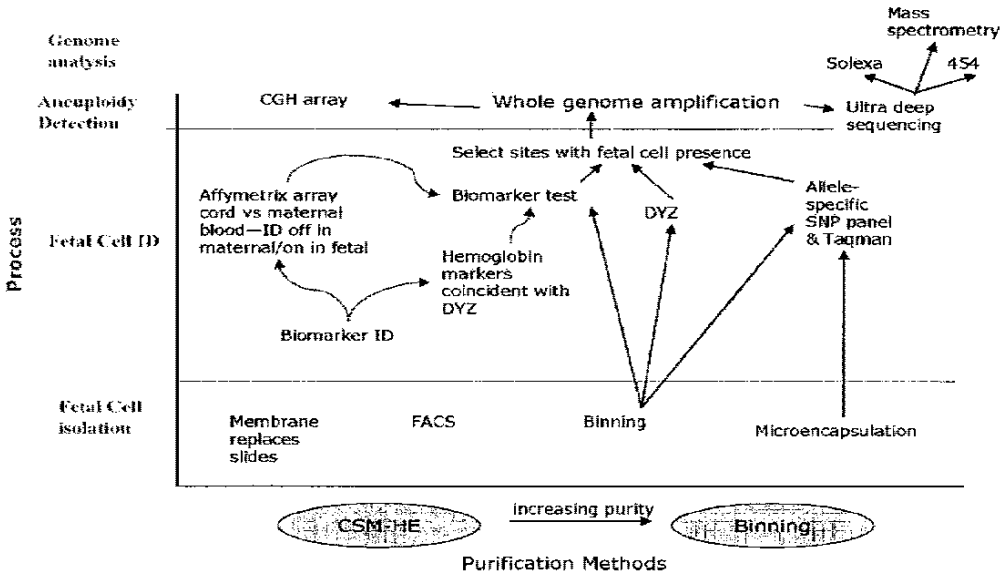

Genome analysis
Aneuploidy Detection
Fetal Cell ID
Fetal Cell isolation
Process
CGH array
Whole genome amplification
Mass spectrometry
Solexa
454
Ultra deep sequencing
Select sites with fetal cell presence
Affymetrix array cord vs maternal blood—ID off in maternal/on in fetal
Biomarker test
DYZ
Allele-specific SNP panel & Taqman
Hemoglobin markers coincident with DYZ
Biomarker ID
Membrane replaces slides
FACS
Binning
Microencapsulation
CSM-HE
increasing purity
Binning
Purification Methods

FIGURE 35

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(71) Applicant *(for all designated States except US)*: **ILLUMINA, INC.** [US/US]; 9885 Towne Centre Drive, San Diego, CA 92121 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **LINNARSSON, Sten** [SE/SE]; C/o 9885 Towne Centre Drive, San Diego, CA 92121 (US).

(74) Agents: **HEBERT, Michael L.** et al.; Mcdermott Will & Emery LLP, 11682 El Camino Real, Suite 400, San Diego, CA 92130 (US).

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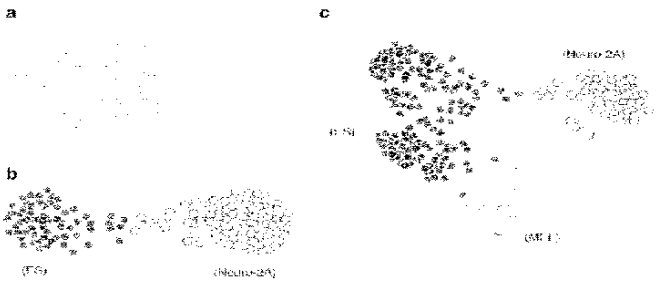


Figure 21

(57) Abstract: The present invention provides methods and compositions for the analysis of gene expression in single cells or in a plurality of single cells. The invention provides methods for preparing a cDNA library from individual cells by releasing mRNA from each single cell to provide a plurality of individual mRNA samples, synthesizing cDNA from the individual mRNA samples, tagging the individual cDNA, pooling the tagged cDNA samples and amplifying the pooled cDNA samples to generate a cDNA library. The invention also provides a cDNA library produced by the methods described herein. The invention farther provides methods for analyzing gene expression in a plurality of cells by preparing a cDNA library as described herein and sequencing the library.

WO 2010/117620 A2

GENE EXPRESSION ANALYSIS IN SINGLE CELLS

The application claims the benefit of priority to United States Provisional application serial No. 61/164,759, filed March 30, 2009, the entire contents of which is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to the analysis of gene expression in single cells. In particular, the invention relates to a method for preparing a cDNA library from a plurality of single cells, and to a cDNA library produced by this method. The cDNA libraries prepared by the method of the invention are suitable for analysis of gene expression by sequencing.

BACKGROUND OF THE INVENTION

The determination of the mRNA content of a cell or tissue (i.e. "gene expression profiling") provides a method for the functional analysis of normal and diseased tissues and organs. For example, gene expression profiling can be used in the study of embryogenesis; for the characterization of primary tumor samples; for the analysis of biopsies from diseased and normal tissue in, for example, psoriasis; for the comparative analysis of cell types from different species to delineate the evolution of development; as an assay system for diagnostics; as a quality control system in cell replacement therapy (i.e. to ensure that a culture of cells is sufficiently pure, and the cells are correctly differentiated); and as an *in vitro* tool to measure the effect of a transfected gene or siRNA on downstream targets in spite of less than 100% transfection efficiency.

Gene expression profiling is usually performed by isolating mRNA from tissue samples and subjecting this mRNA to microarray hybridization. However, such methods only allow previously known genes to be analyzed, and cannot be used to analyze alternative splicing, promoters and polyadenylation signals.

Therefore, direct sequencing of the all, or parts, of the mRNA content of a tissue is being increasingly used (Cloonan et al., Nat Methods 5(7):613-9 (2008)). However, current methods of analyzing the mRNA content of cells by direct sequencing rely on analyzing bulk mRNA obtained from tissue samples typically containing millions of cells. This means that much of the functional information present in single cells is lost or blurred when gene expression is analyzed in bulk mRNA. In addition, dynamic processes, such as the cell cycle,

cannot be observed in population averages. Similarly, distinct cell types in a complex tissue (e.g. the brain) can only be studied if cells are analyzed individually.

Gene expression in single cells has previously been analyzed using a variety of methods (see, for example, Brail et al., Mutat Res 406(2-4):45-54 (1999) ; Levsky et al., Science 297(5582):836-40 (2002); Bengtsson et al. Genome Res 15(10):1388-92 (2005); Esumi et al., Nat Genet 37(2):171-6 (2005). In particular, single cell gene expression in neural cells has been studied by microarray analysis (see Esumi et al. Neurosci Res 60(4):439-51 (2008)). However, these methods require that each single cell is analyzed individually and treated separately during the entire procedure, which is time-consuming and expensive. In addition, the preparation and amplification of samples from single cells independently potentially introduces cell-to-cell variation. Furthermore, as the cDNA of each cell must be amplified to an amount that can be reasonably handled for the subsequent analysis, there is potential amplification bias. For example, a single cell contains about 0.3 pg of mRNA, and at least a 300 ng is commonly needed for subsequent analysis by sequencing. Therefore, an amplification of at least a million-fold is required.

Additionally, microarrays have two major shortcomings: they are linked to known genes, and they have limited sensitivity and dynamic range. RNA sequencing (RNA-Seq) overcomes these problems by sequencing RNA directly (Ozsolak et al., Nature 461:814-818 (2009)) or after reverse-transcription to cDNA (Cloonan et al., Nat. Methods 5:613-619 (2008); Mortazavi et al., Nat. Methods 5:621-628 (2008); Wang et al., Nature 456:470-476 (2008)). Sequence reads are mapped to the genome to reveal sites of transcription, and quantitation is based simply on hit counts, with great sensitivity and nearly unlimited dynamic range.

Tissues are rarely homogeneous, however, and therefore any expression profile based on a tissue sample, biopsy or cell culture will confound the true expression profiles of its constituent cells. One way of getting around this problem would be to analyze single cells instead of cell populations, and indeed single-cell methods have been developed for both microarrays (Esumi et al., Neurosci. Res. 60:439-451 (2008) and Kurimoto et al., Nucleic Acids Res. 34:42 (2006)). These methods are suitable for the analysis of small numbers of single cells, and in particular may be used to study cells that are difficult to obtain in large numbers, such as oocytes and the cells of the early embryo. Cells may be isolated for example by laser capture microdissection, or by microcapillary, and marker genes may be used to locate cells of interest. However, single-cell transcriptomics must confront two great challenges. First, markers suitable for the prospective isolation of defined cell populations are not available for every cell

type, reflecting the fact that few cell types are clearly defined in molecular terms. Second, transcript abundances vary greatly from cell to cell. For example, (β-Actin (Actb) mRNA content varies more than three orders of magnitude between pancreatic islets cells (Bengtsson et al., Genome Res. 15:1388-1392 (2005)). Similar results have been reported, using a variety of detection methods, for RNA polymerase II (Raj et al., PLoS Biol 4:309 (2006)), GAPDH (Lagunavicius et al., RNA 15:765-771 (2009) and Warren et al., Proc. Natl. Acad. Sci. U.S.A. 103:17807-17812 (2006)), PU.1 (Warren et al., *supra*), and TBP, B2M, SDHA and EEIFG mRNAs (Taniguchi et al., Nat Methods 6:503-506 (2009)), and at present seems to be a common feature of the transcriptome.

Most of the variation may be intrinsic, caused by burst-like stochastic activation of transcription, where brief episodes of mRNA synthesis lasting a few minutes are separated by periods of transcriptional silence of similar duration (Chubb et al., Curr Biol 16:1018-1025 (2006)). Each burst would give rise to a dense population of mRNA in the nucleus, which is then exported to the cytoplasm and rapidly decays. As a consequence, a random sample of cells would show great variation in their content of particular mRNAs, ranging from those cells that have just undergone a burst, to those that have nearly completely degraded their mRNA; this has been directly observed for RNA polymerase II transcription *in situ* using a fluorescent probe targeting the 52-copy repeat in that gene (Raj et al., PLoS Biol 4:309 (2006)).

In summary, there are often no suitable cell-surface markers to use in isolating single cells for study, and even when there are, a small number of single cells is not sufficient to capture the range of natural variation in gene expression. The present invention aims to overcome, or reduce, these problems by providing a method of preparing cDNA libraries which can be used to analyze gene expression in a plurality of single cells.

SUMMARY OF THE INVENTION

The invention provides method for preparing a cDNA library from a plurality of single cells. In one aspect, the method includes the steps of releasing mRNA from each single cell to provide a plurality of individual mRNA samples, synthesizing a first strand of cDNA from the mRNA in each individual mRNA sample and incorporating a tag into the cDNA to provide a plurality of tagged cDNA samples, pooling the tagged cDNA samples and amplifying the pooled cDNA samples to generate a cDNA library having double-stranded cDNA. The invention also provides a cDNA library produced by the methods described herein. The

invention further provides methods for analyzing gene expression in a plurality of cells by preparing a cDNA library as described herein and sequencing the library.

BRIEF DESCRIPTION OF THE DRAWINGS

The figures are intended to illustrate broad concepts of the invention by reference to representative examples for ease of discussion. They are not intended to limit the scope of the invention by showing one out of several alternate embodiments or by showing or omitting optional features of the invention.

Figure 1, panels A-F, show an overview of a method of analyzing gene expression in a plurality of single cells. (A) the tissue of interest is dissected; (B) a plurality of single cells are selected; (C) single cells are placed in separate wells of a 96-well plate and lysed; tagged reverse transcription is performed on each sample to produce cDNA; (D) cDNA samples are pooled and amplified; (E) sequencing is performed to obtain 100 million reads; and (F) identification of expressed genes and identification of cells from which they originated.

Figure 2 shows the synthesis of cDNA by template switching.

Figure 3 shows an example of a template switching oligonucleotide comprising a 5' amplification primer sequence (APS), a cell tag and a 3' sequence for template switching.

Figure 4 shows an example of a cDNA synthesis primer (CDS) comprising a 5' amplification primer sequence (APS), a cell tag and a 3' RNA complementary sequence (RCS).

Figure 5 shows the visualization of cDNA samples L001 and L002 following full-length cDNA amplification by PCR. Lane 1: 100 bp marker ladder; lanes 2-3: 25 cycles; lanes 4-5: 30 cycles; lanes 6-7: 35 cycles. Even lanes contain sample L001 and odd lanes contain sample L002.

Figure 6 shows a dilution series from a test PCR using sample L001 (lanes 3-10). Lanes 2 and 11 contain a 100 bp ladder as a size marker.

Figure 7, panels A and B, show the gel electrophoresis separation and isolation of cDNA libraries. Panel A shows a cDNA library following final amplification by PCR (16 cycles) (lanes 5 and 6). Panel B shows the 125-200 bp region has been excised. Lanes 3 and 8 contain 100 bp ladder as a size marker.

Figure 8 shows an example of a sequenced cDNA molecule from a tagged cDNA library. The primer sequences for SOLiD sequencing (P1 and P2) are underlined. The cell-specific tag is boxed. The 2-5 Gs from the template-switching mechanism are shaded by a grey box. Sequence from the TOPO cloning vector is shown in italics. The insert in this case is tubulin beta 2c.

Figure 9 shows a graphical representation of results from a quantitative real-time PCR comparing bulk cDNA (horizontal axis) *versus* 96-cell tagged cDNA (vertical axis). Each circle represents a PCR primer pair directed against the indicated genes. The units are arbitrary and are derived from the cycles-to-threshold value, C_T.

Figure 10, panels A-E, show an overview of the single-cell tagged reverse transcription (STRT) method and exemplary results. (A) Overview of the method, illustrating how single cells were tracked. Well-specific (and hence cell-specific) barcodes were incorporated during cDNA synthesis, resulting in a library where each molecule carried a barcode identifying the cell of origin. (B) Example of reads mapped to both strands of the 5 kb Pou5f1 locus, shown as a coverage plot. Reads were strand-specific and mapped mostly to exons. Non-mRNA background was minimal as judged by hits to introns on the forward strand (top lane) or to the reverse strand (bottom lane). (C) Comparison with standard RNA-Seq (Cloonan et al. Nat. Methods 5:613-619 (2008)) for the 6 kb Nanog locus. Since cDNA synthesis was primed from the poly(A) tail, reads were typically clustered at the 3' end (top lane). The extended 3' UTR of Nanog (light blue) was clearly detected by both methods. SQRL reads extended to 5' exons, but showed more intronic background. (D) The mitochondrial genome. As expected, transcription was nearly exclusively detected on the H strand (top), with only a few truncated transcripts (arrow) originating on the L strand. Protein-coding genes are indicated on the bottom. (E) Gene expression on chromosome 19. Coverage on the forward (top lane) and reverse (middle) strands was highly correlated with local gene density (bottom). Genes are shown as horizontal stacked bars. For clarity, the vertical scales in (B-E) are truncated at half maximal values.

Figure 11, panels A-F, show graphical representation of steps optionally used in the STRT method. Panel A shows a reverse transcription step. A tailed oligo-dT primer directs synthesis of a cDNA strand. When the end of the template RNA is reached, reverse transcriptase adds 3-4 Cs at 3' of cDNA strand (due to its terminal transferase activity). Panel B shows a template switching step. Barcoded helper oligo anneals transiently, and cDNA synthesis proceeds using the oligo as template. As a consequence, the 3' end of the cDNA will carry barcode (XXXX),

BtsCI recognition (Bts) and primer annealing (Pr) sequences. Panel C shows a single primer PCR step. The ends of the cDNA have identical sequences and are amplified using a single PCR primer, which helps suppress short amplicons. Panel D shows a fragmentation step. The amplified library is fragmented to 200 - 300 bp using controlled DNase digestion. Panel E shows an immobilization and end-repair step. Both 5' (with barcode (Br) and BtsCI site (Bts)) and 3' fragments are bound to beads, while internal fragments are washed away. Panel F shows fragment release and adapter ligation. 5' fragments are released by BtsCI digestion, leaving just the barcode (Bc) and insert (white area). 3' fragments remain stuck on the beads. Genome Analyzer paired-end compatible adapters (P1 and P2) are ligated. The library is sequenced from the P1 primer (and could optionally be sequenced also from P2). P1 reads begin with a 5 bp barcode, followed by 3-4 Gs, followed by the cDNA insert. P2 reads would yield only cDNA sequence.

Figure 12, panels A-D, show a graphical representation of an absence of motifs surrounding the template-switching site. All reads were examined in sample 1 006 for the presence of any motif around the template-switching site (that is, around the 5' end of each read). Sequence logos are shown for the 20 bases of genomic sequence upstream and downstream of the first nucleotide (arrow) of each mapped read. As exemplified in A and B, in typical cases (92 of 96), no strong motif was detected, indicating an absence of significant mispriming events, which would have generated an upstream motif complementary to the primer. In four cases (C and D), there was a general preference for T-rich sequences particularly in the first 20 bases of the read. This occurred in wells with very small numbers of reads, indicating a failed reaction. However, in a single case the T-rich motif was observed despite a large number of reads.

Figure 13, panels A and B, show a graphical representation of hotspots for template switching. (A) The Actb locus is expressed from the lower (reverse) strand, right to left in the figure. The top two tracks show aggregate hits on the forward and reverse strands, respectively, demonstrating strand specificity and lack of background in introns. The middle (blue) track shows the exon/intron structure of the gene. The lower track shows individual hits from single cells. Each pixel row represents the hits from a single cell as black dots. There are 96 pixel rows altogether. (B) The same analysis was done for Sox2, a single-exon gene transcribed on the forward (upper) strand, showing the usual 3' bias. In both (A) and (B), hotspots were clearly visible, and were shared among cells, suggesting they represent structural sites on mRNA that favor termination of cDNA synthesis, RNA hydrolysis and/or template switching.

Figure 14, panels A and B, show a graphical representation of the new discovery rate. (A) shows the rate of discovery of distinct mapping reads as a function of the total number of reads. None of the samples were sequenced to saturation, and most libraries would likely contain at least 3 million distinct molecules, indicating that on average at least 30,000 distinct molecules per cell were successfully converted to amplifiable cDNA. The curves are wiggly due to inhomogeneities in the data, presumably caused by imperfections in the cluster PCR process that can generate local duplicates and thus less than random sampling. In (B) the rate of discovery of distinct annotated features is shown as a function of the number of mapped reads (for sample L006). Saturation was reached quickly, showing that most features present in the sample could be discovered at modest sampling depth.

Figure 15 shows a graphical representation for distinguishing expression of overlapping genes. Because of the strand-specificity of the template-switching mechanism, strandedness could be maintained throughout the protocol. This was especially important for genes with overlapping exons. Represented in the figure is an example of such a pair of genes, Cathepsin A (Ctsa) and Phospholipid transfer protein (Pltp), whose last exons overlap. Without strand information, the reads in the last four exons of Pltp could not be distinguished from the reads originating in the last exon of Ctsa. There are about 3,000 genes with similar overlapping 3' exons.

Figure 16 shows a graphical representation of length bias for transcripts. In order to detect any bias against short or long transcripts, the average expression level was calculated as a function of mRNA length (in 200 bp bins) for sample L019. Each bar shows the expression level of genes with transcripts shorter than the indicated length (thus the first bar contains transcripts 0 - 200 bp long). Over a wide range of mRNA length, there was no apparent difference in measured expression levels. The shortest transcripts (<200 bp) were presumably suppressed by the gel purification step where inserts >100 bp were selected. The apparent overexpression of genes in the 400 - 800 bp range can possibly be explained by a more efficient template switching in this range, where cDNA synthesis would often reach the 5' cap of mRNA. Alternatively, it can simply be due to the presence of a few very highly expressed genes in this range, including Dppa5 and Rps14.

Figure 17, panels A-F, show a graphical representation of the quantitative accuracy of the STRT method. (A) Distribution of gene expression levels, in transcripts per million (t.p.m.) showed predominantly low expression, in the 10 - 100 t.p.m. range. (B) Comparison of two cells sequence to a depth of approximately 500 000 reads/cell. In this case, genes below 100 t.p.m. could be accurately quantified. (C) Comparison of two cells sequenced to

approximately 100 000 reads/cell. In this case, sensitivity dropped to about 1000 t.p.m. (D) Probability of detection as a function of expression level. Each dot shows a gene, with a given average expression level (across all cells) and fraction of cells having non-zero expression of this gene. The distribution approach the theoretical limit of random sampling given the actual depth of sequencing used here (dashed line). (E) Comparison with quantitative real-time PCR for the four ES cells shown in (B) and (C), using select markers of pluripotency and differentiation. In general, the quantitative accuracy was good, with a single putative false positive (Eomes in ES Cell #4). However this was a rare event, and was not observed for this gene in any of the other 160 ES cells examined. Q-PCR levels were converted to t.p.m. by normalization of the Actb/1081 primer pair with ES cells #1 and #2. Actin was then independently measured using a different primer pair (Actb/1832) to confirm the accuracy of the normalization.

Figure 18 shows a graphical comparison between STRT, Q-PCR and microarray analyses. Genes expected to be expressed (Actb, Pou5f1, Zfp42, Sox2, Klf4, Nanog, Plk1, Zic3) or not expressed (Gata4, Brachyury, Eomes, Otx1, Cdx2, Gata5, Calb1, Gfap, Dppa3 and NeuroD1) in undifferentiated ES cells were analyzed by STRT, quantitative real-time PCR (Q-PCR) and Illumina microarray. There was good correlation between STRT and Q-PCR, and to a lesser extent with microarray data. In particular, Sox2 was undercalled on the microarray, while Otx1 and Dppa3 were apparent false positives. The microarray data is the mean of two hybridization reactions. Q-PCR was performed in duplicate and repeated once for confirmation, and the STRT data is the mean of 160 single ES cells.

Figure 19, panels A and B, show a graphical representation of gene expression distribution across cells. (A) shows the distribution of Actb expression levels across all cells using STRT. (B) shows Actb expression in pancreatic islet cells from Bengtsson et al. Genome Res 15(10):1388-92 (2005) for comparison.

Figure 20 shows a principal component analysis. In order to discover and cluster cell types based on expression data, the five independently prepared 96-cell samples were subjected to principal component analysis. The three types of cells (ES, Neuro-2A and MEF) clearly clustered separately, although MEFs did not form a very distinct cluster. Moreover, ES cells prepared independently clustered together, showing that the PCA did not simply pick up sample preparation differences. This demonstrates that single-cell expression data can be used to accurately classify cell types.

Figure 21, panels A-C, show a graph-based visualization ("cell map") of the expression pattern. (A) Cells, represented by graph nodes (circles) were laid out randomly, and edges were drawn from each cell to the five other cells it was most highly correlated with. (B) Force-directed layout was used to lay out the graph on a plane. In this layout, cells repelled each other uniformly, but were held together by edges acting as elastic springs. The resulting visual map was consistent with known cell identities (ES cells vs Neuro-2A cells), with some less deeply sequenced cells showing poor separation. (C) Adding more ES cells as well as fibroblasts (MEFs) expanded the map and demonstrated that independently prepared ES cells grouped together accurately.

Figure 22 shows the visualizing of gene expression on a cell map of **Figure 21**. Each map retains its layout from **Figure 21C**, but cells are shaded according to expression of the indicated gene. A logarithmic scale was used (upper right). Mitochondrial ribosomal RNA 2 (mt_Rnr2) was the highest-expressed gene of all. Housekeeping genes such as Actin (Actb) and ribosomal protein L4 (Rpl4) were detected in all cell types, but not in every single cell. The power of shotgun single cell expression profiling was revealed for low-expressed genes like K-ras (Kras), which was detected only in approximately half of all cells, but still clearly expressed in all cell types. Calbindin (Calb1) was absent, as expected and confirmed by Q-PCR. A set of well-known ES cell markers (Dppa5, Sox2, Sall4, Pou5f1, Nanog, Zfp42, Zic3 and Esrrb) were clearly expressed specifically in the ES cell cluster, whereas Klf4, Myc and Klf2 were more widely distributed. Dppa3 was not detected, as confirmed by Q-PCR (**Figure 18**)

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions for the analysis of gene expression in single cells or in a plurality of single cells. In particular, the invention provides methods for preparing a cDNA library from a plurality of single cells. The methods are based on determining gene expression levels from a population of individual cells, which can be used to identify natural variations in gene expression on a cell by cell level. The methods can also be used to identify and characterize the cellular composition of a population of cells in the absence of suitable cell-surface markers. The methods described herein also provide the advantage of generating a cDNA library representative of RNA content in a cell population by using single cells, whereas cDNA libraries prepared by classical methods typically require total RNA isolated from a large population (see Example I). Thus, a cDNA library produced using the methods of the invention provide at least equivalent representation of RNA content in a

population of cells by utilizing a smaller subpopulation of individual cells along with additional advantages as described herein.

Embodiments of the invention also provide sampling of a large number of single cells. Using similarity of expression patterns, a map of cells can be built showing how the cells relate. This map can be used to distinguish cell types *in silico*, by detecting clusters of closely related cells (see Example II). By sampling not just a few, but large numbers of single cells, similarity of expression patterns can be used to build a map of cells and how they are related. This method permits access to undiluted expression data from every distinct type of cell present in a population, without the need for prior purification of those cell types. In addition, where known markers are available, these can be used *in silico* to delineate cells of interest. The validity of this approach is shown in Example II, which analyzes a collection of cells sampled from three distinct cell types (mouse embryonic stem cells, embryonic fibroblasts and neuroblastoma cells) of distinct embryonic origins (pluripotent stem cells vs. mesodermal and ectodermal germ layers) and disease state (normal vs. transformed).

Embodiments of the invention provide a method of preparing a cDNA library from a plurality of single cells by releasing mRNA from each single cell to provide a plurality of individual samples, wherein the mRNA in each individual mRNA sample is from a single cell, synthesizing a first strand of cDNA from the mRNA in each individual mRNA sample and incorporating a tag into the cDNA to provide a plurality of tagged cDNA samples, wherein the cDNA in each tagged cDNA sample is complementary to mRNA from a single cell pooling the tagged cDNA samples and amplifying the pooled cDNA samples to generate a cDNA library comprising double-stranded cDNA. By utilizing the above method, it is feasible to prepare samples for sequencing from several hundred single cells in a short time and with a minimal amount of work. Traditional methods for preparing a fragment library from RNA for sequencing include gel excision steps that are laborious. In the absence of special equipment, it is not convenient to prepare more than a handful of samples in parallel. In some aspects of the methods described herein, a set of 96 cells is prepared as a single sample (after cDNA synthesis), which makes it feasible to prepare several hundred cells for sequencing. Additionally, technical variation is minimized because each set of 96 cells is prepared together (in a single tube).

In some aspects of the invention, each cDNA sample obtained from a single cell is tagged, which allows gene expression to be analyzed at the level of a single cell. This allows dynamic processes, such as the cell cycle, to be studied and distinct cell types in a complex tissue (e.g.

the brain) to be analyzed. In some aspects of the invention, the cDNA samples can be pooled prior to analysis. Pooling the samples simplifies handling of the samples from each single cell and reduces the time required to analyze gene expression in the single cells, which allows for high throughput analysis of gene expression. Pooling of the cDNA samples prior to amplification also provides the advantage that technical variation between samples is virtually eliminated. In addition, as the cDNA samples are pooled before amplification, less amplification is required to generate sufficient amounts of cDNA for subsequent analysis compared to amplifying and treating cDNA samples from each single cell separately. This reduces amplification bias, and also means that any bias will be similar across all the cells used to provide pooled cDNA samples. RNA purification, storage and handling are also not required, which helps to eliminate problems caused by the unstable nature of RNA.

As the cDNA libraries produced by the method of the invention are suitable for the analysis the gene expression profiles of single cells by direct sequencing, it is possible to use these libraries to study the expression of genes which were not previously known, and also to analyze alternative splicing, promoters and polyadenylation signals. Preparing the cDNA libraries as described herein, provides for a sensitive method for detecting a single or low copy RNA transcript. The sensitivity of the method is shown in **Figure 17D** and described in Example II. For example, genes expressed at 100 transcripts per million (t.p.m.) are detected about 50% of the time. However, as shown in **Figure 14A**, the samples were not saturated, so there is additional sensitivity that is achievable with deeper sequencing of the samples. Accordingly, the method for preparing the cDNA libraries as described herein detect a single or low copy RNA transcript at least 30% of the time, or alternatively at least 40% of the time, or at least 50% of the time, or alternatively at least 60% of the time, or alternatively at least 70 % of the time, or alternatively at least 80% of the time or alternatively at least 90% of the time, or alternatively at least 95% of the time.

Embodiments of the invention also provide a method for identifying a single cell type out of a sample and/or determining the transcriptome of a single cell by preparing a cDNA library as described herein, determining the expression levels of individual cells in a population, and mapping of the individual cells based on similarity of expression patterns. Mapping of individual cells can be done *in silico* by one of skill in the art and in particular utilizing the methods described herein, such as shown in Example II. The number of cells needed to determine the frequency of a given cell type in the plurality of cells will follow a binomial distribution. For example, a predetermined number of individual cells can be sampled so that

at least ten of the desired type are expected to be detected. Accordingly, if the frequency of the cell type in the sample is 10%, a cDNA library from approximately 100 cells will need to be prepared and analyzed as described herein.

The term "cDNA library" refers to a collection of cloned complementary DNA (cDNA) fragments, which together constitute some portion of the transcriptome of a single cell or a plurality of single cells. cDNA is produced from fully transcribed mRNA found in a cell and therefore contains only the expressed genes of a single cell or when pooled together the expressed genes from a plurality of single cells.

As used herein, a "plurality" refers to a population of cells and can include any number of cells desired to be analyzed. In some aspects of the invention, a plurality of cells includes at least 10 cells, or alternatively at least 25 cells, or alternatively at least 50 cells, or alternatively at least 100 cells, or alternatively at least 200 cells, or alternatively at least 500 cells, or alternatively at least 1000 cells, or alternatively 5,000 cells or alternatively 10,000 cells. In another aspect of the invention, a plurality of cells includes from 10 to 100 cells, or alternatively from 50 to 200 cells, alternatively from 100 to 500 cells, or alternatively from 100 to 1000, or alternatively from 1,000 to 5,000 cells.

The expression "amplification" or "amplifying" refers to a process by which extra or multiple copies of a particular polynucleotide are formed. Amplification includes methods such as PCR, ligation amplification (or ligase chain reaction, LCR) and amplification methods. These methods are known and widely practiced in the art. See, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202 and Innis et al., "PCR protocols: a guide to method and applications" Academic Press, Incorporated (1990) (for PCR); and Wu et al. (1989) Genomics 4:560-569 (for LCR). In general, the PCR procedure describes a method of gene amplification which is comprised of (i) sequence-specific hybridization of primers to specific genes within a DNA sample (or library), (ii) subsequent amplification involving multiple rounds of annealing, elongation, and denaturation using a DNA polymerase, and (iii) screening the PCR products for a band of the correct size. The primers used are oligonucleotides of sufficient length and appropriate sequence to provide initiation of polymerization, i.e. each primer is specifically designed to be complementary to each strand of the genomic locus to be amplified.

Reagents and hardware for conducting amplification reaction are commercially available. Primers useful to amplify sequences from a particular gene region are preferably complementary to, and hybridize specifically to sequences in the target region or in its flanking

regions and can be prepared using the polynucleotide sequences provided herein. Nucleic acid sequences generated by amplification can be sequenced directly.

When hybridization occurs in an antiparallel configuration between two single-stranded polynucleotides, the reaction is called "annealing" and those polynucleotides are described as "complementary". A double-stranded polynucleotide can be complementary or homologous to another polynucleotide, if hybridization can occur between one of the strands of the first polynucleotide and the second. Complementarity or homology (the degree that one polynucleotide is complementary with another) is quantifiable in terms of the proportion of bases in opposing strands that are expected to form hydrogen bonding with each other, according to generally accepted base-pairing rules.

As used herein, a "single cell" refers to one cell. Single cells useful in the methods described herein can be obtained from a tissue of interest, or from a biopsy, blood sample, or cell culture. Additionally, cells from specific organs, tissues, tumors, neoplasms, or the like can be obtained and used in the methods described herein. Furthermore, in general, cells from any population can be used in the methods, such as a population of prokaryotic or eukaryotic single celled organisms including bacteria or yeast. In some aspects of the invention, the method of preparing the cDNA library can include the step of obtaining single cells. A single cell suspension can be obtained using standard methods known in the art including, for example, enzymatically using trypsin or papain to digest proteins connecting cells in tissue samples or releasing adherent cells in culture, or mechanically separating cells in a sample. Single cells can be placed in any suitable reaction vessel in which single cells can be treated individually. For example a 96-well plate, such that each single cell is placed in a single well.

Methods for manipulating single cells are known in the art and include fluorescence activated cell sorting (FACS), micromanipulation and the use of semi-automated cell pickers (e.g. the Quixell™ cell transfer system from Stoelting Co.). Individual cells can, for example, be individually selected based on features detectable by microscopic observation, such as location, morphology, or reporter gene expression.

In some aspects of the invention, mRNA can be released from the cells by lysing the cells. Lysis can be achieved by, for example, heating the cells, or by the use of detergents or other chemical methods, or by a combination of these. However, any suitable lysis method known in the art can be used. A mild lysis procedure can advantageously be used to prevent the release of nuclear chromatin, thereby avoiding genomic contamination of the cDNA library, and to

minimise degradation of mRNA. For example, heating the cells at 72°C for 2 minutes in the presence of Tween-20 is sufficient to lyse the cells while resulting in no detectable genomic contamination from nuclear chromatin. Alternatively, cells can be heated to 65°C for 10 minutes in water (Esumi et al., Neurosci Res 60(4):439-51 (2008)); or 70°C for 90 seconds in PCR buffer II (Applied Biosystems) supplemented with 0.5% NP-40 (Kurimoto et al., Nucleic Acids Res 34(5):e42 (2006)); or lysis can be achieved with a protease such as Proteinase K or by the use of chaotropic salts such as guanidine isothiocyanate (U.S. Publication No. 2007/0281313).

Synthesis of cDNA from mRNA in the methods described herein can be performed directly on cell lysates, such that a reaction mix for reverse transcription is added directly to cell lysates. Alternatively, mRNA can be purified after its release from cells. This can help to reduce mitochondrial and ribosomal contamination. mRNA purification can be achieved by any method known in the art, for example, by binding the mRNA to a solid phase. Commonly used purification methods include paramagnetic beads (e.g. Dynabeads). Alternatively, specific contaminants, such as ribosomal RNA can be selectively removed using affinity purification.

cDNA is typically synthesized from mRNA by reverse transcription. Methods for synthesizing cDNA from small amounts of mRNA, including from single cells, have previously been described (Kurimoto et al., Nucleic Acids Res 34(5):e42 (2006); Kurimoto et al., Nat Protoc 2(3):739-52 (2007); and Esumi et al., Neurosci Res 60(4):439-51 (2008)). In order to generate an amplifiable cDNA, these methods introduce a primer annealing sequence at both ends of each cDNA molecule in such a way that the cDNA library can be amplified using a single primer. The Kurimoto method uses a polymerase to add a 3' poly-A tail to the cDNA strand, which can then be amplified using a universal oligo-T primer. In contrast, the Esumi method uses a template switching method to introduce an arbitrary sequence at the 3' end of the cDNA, which is designed to be reverse complementary to the 3' tail of the cDNA synthesis primer. Again, the cDNA library can be amplified by a single PCR primer. Single-primer PCR exploits the PCR suppression effect to reduce the amplification of short contaminating amplicons and primer-dimers (Dai et al., J Biotechnol 128(3):435-43 (2007)). As the two ends of each amplicon are complementary, short amplicons will form stable hairpins, which are poor templates for PCR. This reduces the amount of truncated cDNA and improves the yield of longer cDNA molecules.

In some aspects of the invention, the synthesis of the first strand of the cDNA can be directed by a cDNA synthesis primer (CDS) that includes an RNA complementary sequence (RCS). In some aspects of the invention, the RCS is at least partially complementary to one or more mRNA in an individual mRNA sample. This allows the primer, which is typically an oligonucleotide, to hybridize to at least some mRNA in an individual mRNA sample to direct cDNA synthesis using the mRNA as a template. The RCS can comprise oligo (dT), or be gene family-specific, such as a sequence of nucleic acids present in all or a majority related genes, or can be composed of a random sequence, such as random hexamers. To avoid the CDS priming on itself and thus generating undesired side products, a non-self-complementary semi-random sequence can be used. For example, one letter of the genetic code can be excluded, or a more complex design can be used while restricting the CDS to be non-self-complementary.

The terms "oligonucleotide" and "polynucleotide" are used interchangeably and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides or analogs thereof. Polynucleotides can have any three-dimensional structure and can perform any function, known or unknown. The following are non-limiting examples of polynucleotides: a gene or gene fragment (for example, a probe, primer, EST or SAGE tag), exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers. A polynucleotide can comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. The term also refers to both double- and single-stranded molecules. Unless otherwise specified or required, any embodiment of this invention that comprises a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

A polynucleotide is composed of a specific sequence of four nucleotide bases: adenine (A); cytosine (C); guanine (G); thymine (T); and uracil (U) for thymine when the polynucleotide is RNA. Thus, the term polynucleotide sequence is the alphabetical representation of a polynucleotide molecule. This alphabetical representation can be input into databases in a computer having a central processing unit and used for bioinformatics applications such as functional genomics and homology searching.

A "primer" is a short polynucleotide, generally with a free 3'-OH group that binds to a target or template potentially present in a sample of interest by hybridizing with the target, and thereafter promoting polymerization of a polynucleotide complementary to the target. Primers

of the instant invention are comprised of nucleotides ranging from 17 to 30 nucleotides. In one aspect, the primer is at least 17 nucleotides, or alternatively, at least 18 nucleotides, or alternatively, at least 19 nucleotides, or alternatively, at least 20 nucleotides, or alternatively, at least 21 nucleotides, or alternatively, at least 22 nucleotides, or alternatively, at least 23 nucleotides, or alternatively, at least 24 nucleotides, or alternatively, at least 25 nucleotides, or alternatively, at least 26 nucleotides, or alternatively, at least 27 nucleotides, or alternatively, at least 28 nucleotides, or alternatively, at least 29 nucleotides, or alternatively, at least 30 nucleotides, or alternatively at least 50 nucleotides, or alternatively at least 75 nucleotides or alternatively at least 100 nucleotides.

The RCS can also be at least partially complementary to a portion of the first strand of cDNA, such that it is able to direct the synthesis of a second strand of cDNA using the first strand of the cDNA as a template. Thus, following first strand synthesis, an RNase enzyme (e.g. an enzyme having RNaseH activity) can be added after synthesis of the first strand of cDNA to degrade the RNA strand and to permit the CDS to anneal again on the first strand to direct the synthesis of a second strand of cDNA. For example, the RCS could comprise random hexamers, or a non-self complementary semi-random sequence (which minimizes self-annealing of the CDS).

A template-switching oligonucleotide (TSO) that includes a portion which is at least partially complementary to a portion of the 3' end of the first strand of cDNA can be added to each individual mRNA sample in the methods described herein. Such a template switching method is described in (Esumi et al., Neurosci Res 60(4):439-51 (2008)) and allows full length cDNA comprising the complete 5' end of the mRNA to be synthesized. As the terminal transferase activity of reverse transcriptase typically causes 2-5 cytosines to be incorporated at the 3' end of the first strand of cDNA synthesized from mRNA, the first strand of cDNA can include a plurality of cytosines, or cytosine analogues that base pair with guanosine, at its 3' end (see US 5,962,272). In one aspect of the invention, the first strand of cDNA can include a 3' portion comprising at least 2, at least 3, at least 4, at least 5 or 2, 3, 4, or 5 cytosines or cytosine analogues that base pair with guanosine. A non-limiting example of a cytosine analogue that base pairs with guanosine is 5-aminoallyl-2'-deoxycytidine.

In one aspect of the invention, the TSO can include a 3' portion comprising a plurality of guanosines or guanosine analogues that base pair with cytosine. Non-limiting examples of guanosines or guanosine analogues useful in the methods described herein include, but are not limited to, deoxyriboguanosine, riboguanosine, locked nucleic acid-guanosine, and peptide

nucleic acid-guanosine. The guanosines can be ribonucleosides or locked nucleic acid monomers.

A locked nucleic acid (LNA) is a modified RNA nucleotide. The ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' oxygen and 4' carbon. The bridge "locks" the ribose in the 3'-endo (North) conformation. Some of the advantages of using LNAs in the methods of the invention include increasing the thermal stability of duplexes, increased target specificity and resistance from exo- and endonucleases.

A peptide nucleic acid (PNA) is an artificially synthesized polymer similar to DNA or RNA, wherein the backbone is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. The backbone of a PNA is substantially non-ionic under neutral conditions, in contrast to the highly charged phosphodiester backbone of naturally occurring nucleic acids. This provides two non-limiting advantages. First, the PNA backbone exhibits improved hybridization kinetics. Secondly, PNAs have larger changes in the melting temperature (Tm) for mismatched versus perfectly matched basepairs. DNA and RNA typically exhibit a 2-4° C drop in Tm for an internal mismatch. With the non-ionic PNA backbone, the drop is closer to 7-9° C. This can provide for better sequence discrimination. Similarly, due to their non-ionic nature, hybridization of the bases attached to these backbones is relatively insensitive to salt concentration.

A nucleic acid useful in the invention can contain a non-natural sugar moiety in the backbone. Exemplary sugar modifications include but are not limited to 2' modifications such as addition of halogen, alkyl, substituted alkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, SO_2CH_3, OSO_2, SO_3, CH_3, ONO_2, NO_2, N_3, NH_2, substituted silyl, and the like. Similar modifications can also be made at other positions on the sugar, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Nucleic acids, nucleoside analogs or nucleotide analogs having sugar modifications can be further modified to include a reversible blocking group, peptide linked label or both. In those embodiments where the above-described 2' modifications are present, the base can have a peptide linked label.

A nucleic acid used in the invention can also include native or non-native bases. In this regard a native deoxyribonucleic acid can have one or more bases selected from the group consisting of adenine, thymine, cytosine or guanine and a ribonucleic acid can have one or more bases selected from the group consisting of uracil, adenine, cytosine or guanine. Exemplary non-

native bases that can be included in a nucleic acid, whether having a native backbone or analog structure, include, without limitation, inosine, xathanine, hypoxathanine, isocytosine, isoguanine, 5-methylcytosine, 5-hydroxymethyl cytosine, 2-aminoadenine, 6-methyl adenine, 6-methyl guanine, 2-propyl guanine, 2-propyl adenine, 2-thiouracil, 2-thiothymine, 2-thiocytosine, 15 -halouracil, 15 -halocytosine, 5-propynyl uracil, 5-propynyl cytosine, 6-azo uracil, 6-azo cytosine, 6-azo thymine, 5-uracil, 4-thiouracil, 8-halo adenine or guanine, 8-amino adenine or guanine, 8-thiol adenine or guanine, 8-thioalkyl adenine or guanine, 8-hydroxyl adenine or guanine, 5-halo substituted uracil or cytosine, 7-methylguanine, 7-methyladenine, 8-azaguanine, 8-azaadenine, 7-deazaguanine, 7-deazaadenine, 3-deazaguanine, 3-deazaadenine or the like. A particular embodiment can utilize isocytosine and isoguanine in a nucleic acid in order to reduce non-specific hybridization, as generally described in U.S. Pat. No. 5,681,702.

A non-native base used in a nucleic acid of the invention can have universal base pairing activity, wherein it is capable of base pairing with any other naturally occurring base. Exemplary bases having universal base pairing activity include 3-nitropyrrole and 5-nitroindole. Other bases that can be used include those that have base pairing activity with a subset of the naturally occurring bases such as inosine, which basepairs with cytosine, adenine or uracil.

In one aspect of the invention, the TSO can include a 3' portion including at least 2, at least 3, at least 4, at least 5, or 2, 3, 4, or 5, or 2-5 guanosines, or guanosine analogues that base pair with cytosine. The presence of a plurality of guanosines (or guanosine analogues that base pair with cytosine) allows the TSO to anneal transiently to the exposed cytosines at the 3' end of the first strand of cDNA. This causes the reverse transcriptase to switch template and continue to synthesis a strand complementary to the TSO. In one aspect of the invention, the 3' end of the TSO can be blocked, for example by a 3' phosphate group, to prevent the TSO from functioning as a primer during cDNA synthesis.

In one aspect of the invention, the mRNA is released from the cells by cell lysis. If the lysis is achieved partially by heating, then the CDS and/or the TSO can be added to each individual mRNA sample during cell lysis, as this will aid hybridization of the oligonucleotides. In some aspects, reverse transcriptase can be added after cell lysis to avoid denaturation of the enzyme.

In some aspects of the invention, a tag can be incorporated into the cDNA during its synthesis. For example, the CDS and/or the TSO can include a tag, such as a particular nucleotide

sequence, which can be at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15 or at least 20 nucleotides in length. For example, the tag can be a nucleotide sequence of 4-20 nucleotides in length, e.g. 4, 5, 6, 7, 8, 9, 10, 15 or 20 nucleotides in length. As the tag is present in the CDS and/or the TSO it will be incorporated into the cDNA during its synthesis and can therefore act as a "barcode" to identify the cDNA. Both the CDS and the TSO can include a tag. The CDS and the TSO can each include a different tag, such that the tagged cDNA sample comprises a combination of tags. Each cDNA sample generated by the above method can have a distinct tag, or a distinct combination of tags, such that once the tagged cDNA samples have been pooled, the tag can be used to identify which single cell from each cDNA sample originated. Thus, each cDNA sample can be linked to a single cell, even after the tagged cDNA samples have been pooled in the methods described herein.

Before the tagged cDNA samples are pooled, synthesis of cDNA can be stopped, for example by removing or inactivating the reverse transcriptase. This prevents cDNA synthesis by reverse transcription from continuing in the pooled samples. The tagged cDNA samples can optionally be purified before amplification, either before or after they are pooled.

The pooled cDNA samples can be amplified by polymerase chain reaction (PCR) including emulsion PCR and single primer PCR in the methods described herein. For example, the cDNA samples can be amplified by single primer PCR. The CDS can comprise a 5' amplification primer sequence (APS), which subsequently allows the first strand of cDNA to be amplified by PCR using a primer that is complementary to the 5' APS. The TSO can also comprise a 5' APS, which can be at least 70% identical, at least 80% identical, at least 90% identical, at least 95% identical, or 70%, 80%, 90% or 100% identical to the 5' APS in the CDS. This means that the pooled cDNA samples can be amplified by PCR using a single primer (i.e. by single primer PCR), which exploits the PCR suppression effect to reduce the amplification of short contaminating amplicons and primer-dimers (Dai et al., J Biotechnol 128(3):435-43 (2007)). As the two ends of each amplicon are complementary, short amplicons will form stable hairpins, which are poor templates for PCR. This reduces the amount of truncated cDNA and improves the yield of longer cDNA molecules. The 5' APS can be designed to facilitate downstream processing of the cDNA library. For example, if the cDNA library is to be analyzed by a particular sequencing method, e.g. Applied Biosystems' SOLiD sequencing technology, or Illumina's Genome Analyzer, the 5' APS can be designed to be identical to the primers used in these sequencing methods. For example, the 5' APS can be

identical to the SOLiD P1 primer, and/or a SOLiD P2 sequence inserted in the CDS, so that the P1 and P2 sequences required for SOLiD sequencing are integral to the amplified library.

Another exemplary method for amplifying pooled cDNA includes PCR. PCR is a reaction in which replicate copies are made of a target polynucleotide using a pair of primers or a set of primers consisting of an upstream and a downstream primer, and a catalyst of polymerization, such as a DNA polymerase, and typically a thermally-stable polymerase enzyme. Methods for PCR are well known in the art, and taught, for example in MacPherson et al. (1991) PCR 1 : A Practical Approach (IRL Press at Oxford University Press). All processes of producing replicate copies of a polynucleotide, such as PCR or gene cloning, are collectively referred to herein as replication. A primer can also be used as a probe in hybridization reactions, such as Southern or Northern blot analyses.

For emulsion PCR, an emulsion PCR reaction is created by vigorously shaking or stirring a "water in oil" mix to generate millions of micron-sized aqueous compartments. The DNA library is mixed in a limiting dilution either with the beads prior to emulsification or directly into the emulsion mix. The combination of compartment size and limiting dilution of beads and target molecules is used to generate compartments containing, on average, just one DNA molecule and bead (at the optimal dilution many compartments will have beads without any target) To facilitate amplification efficiency, both an upstream (low concentration, matches primer sequence on bead) and downstream PCR primers (high concentration) are included in the reaction mix. Depending on the size of the aqueous compartments generated during the emulsification step, up to $3x10^9$ individual PCR reactions per µl can be conducted simultaneously in the same tube. Essentially each little compartment in the emulsion forms a micro PCR reactor. The average size of a compartment in an emulsion ranges from sub-micron in diameter to over a 100 microns, depending on the emulsification conditions.

"Identity," "homology" or "similarity" are used interchangeably and refer to the sequence similarity between two nucleic acid molecules. Identity can be determined by comparing a position in each sequence which can be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous at that position. A degree of identity between sequences is a function of the number of matching or identical positions shared by the sequences. An unrelated or non-homologous sequence shares less than 40% identity, or alternatively less than 25% identity, with one of the sequences of the present invention.

A polynucleotide has a certain percentage (for example, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99%) of "sequence identity" to another sequence means that, when aligned, that percentage of bases are the same in comparing the two sequences. This alignment and the percent sequence identity or homology can be determined using software programs known in the art, for example those described in Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y., (1993). Preferably, default parameters are used for alignment. One alignment program is BLAST, using default parameters. In particular, programs are BLASTN and BLASTP, using the following default parameters: Genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + SwissProtein + SPupdate + PIR. Details of these programs can be found at the National Center for Biotechnology Information.

The method of preparing a cDNA library described herein can further comprise processing the cDNA library to obtain a library suitable for sequencing. As used herein, a library is suitable for sequencing when the complexity, size, purity or the like of a cDNA library is suitable for the desired screening method. In particular, the cDNA library can be processed to make the sample suitable for any high-throughout screening methods, such as Applied Biosystems' SOLiD sequencing technology, or Illumina's Genome Analyzer. As such, the cDNA library can be processed by fragmenting the cDNA library (e.g. with DNase) to obtain a short-fragment 5'-end library. Adapters can be added to the cDNA, e.g. at one or both ends to facilitate sequencing of the library. The cDNA library can be further amplified, e.g. by PCR, to obtain a sufficient quantity of cDNA for sequencing.

Embodiments of the invention provide a cDNA library produced by any of the methods described herein. This cDNA library can be sequenced to provide an analysis of gene expression in single cells or in a plurality of single cells.

Embodiments of the invention also provide a method for analyzing gene expression in a plurality of single cells, the method comprising the steps of preparing a cDNA library using the method described herein and sequencing the cDNA library. A "gene" refers to a polynucleotide containing at least one open reading frame (ORF) that is capable of encoding a particular polypeptide or protein after being transcribed and translated. Any of the polynucleotide sequences described herein can be used to identify larger fragments or full-length coding sequences of the gene with which they are associated. Methods of isolating larger fragment sequences are known to those of skill in the art.

As used herein, "expression" refers to the process by which polynucleotides are transcribed into mRNA and/or the process by which the transcribed mRNA is subsequently being translated into peptides, polypeptides, or proteins. If the polynucleotide is derived from genomic DNA, expression can include splicing of the mRNA in an eukaryotic cell.

The cDNA library can be sequenced by any suitable screening method. In particular, the cDNA library can be sequenced using a high-throughout screening method, such as Applied Biosystems' SOLiD sequencing technology, or Illumina's Genome Analyzer. In one aspect of the invention, the cDNA library can be shotgun sequenced. The number of reads can be at least 10,000, at least 1 million, at least 10 million, at least 100 million, or at least 1000 million. In another aspect, the number of reads can be from 10,000 to 100,000, or alternatively from 100,000 to 1 million, or alternatively from 1 million to 10 million, or alternatively from 10 million to 100 million, or alternatively from 100 million to 1000 million. A "read" is a length of continuous nucleic acid sequence obtained by a sequencing reaction.

"Shotgun sequencing" refers to a method used to sequence very large amount of DNA (such as the entire genome). In this method, the DNA to be sequenced is first shredded into smaller fragments which can be sequenced individually. The sequences of these fragments are then reassembled into their original order based on their overlapping sequences, thus yielding a complete sequence. "Shredding" of the DNA can be done using a number of difference techniques including restriction enzyme digestion or mechanical shearing. Overlapping sequences are typically aligned by a computer suitably programmed. Methods and programs for shotgun sequencing a cDNA library are well know in the art.

An embodiment of the method of the invention is summarized in **Figure 1**. Cells are obtained from a tissue of interest and a single-cell suspension is obtained. A single cell is placed in one well of a 96-well plate in Cell Capture Mix. The cells are lysed and reverse transcription reaction mix is added directly to the lysates without additional purification. This results in the synthesis of cDNA from cellular mRNA and incorporation of a tag into the cDNA. The tagged cDNA samples are pooled and amplified and then sequenced to produce 100 million reads. This allows identification of genes that are expressed in each single cell.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also provided within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

Single-cell tagged reverse transcription (STRT)

An embodiment of the method of the invention may be called "single-cell tagged reverse transcription" (STRT) and is described in detail below.

Cell Collection and Lysis

A 96-well plate containing Cell Capture Mix was made by aliquoting 5 µl/well from the Cell Capture Master Plate (see Table 1 below) into an AbGene Thermo-Fast plate.

Reagent	**For one well**	**For one plate**	**Final concentration**
STRT-T30-BIO (100 µM)	0.25 µL	27.5 µL	400 nM
Reagent	**For one well**	**For one plate**	**Final concentration**
STRT buffer (5x)	12.5 µL	1375 µL	1x
STRT-FW-n (5 µM)	5 µL	(5 µL/well)	400 nM
Water	44.75	4.9 mL	
Total	**62.5 uL**		

Table 1 Making a STRT Cell Capture Master Plate.

27.5 µL STRT-T30- BIO (100 µM) was mixed with 1375 µL STRT 5x buffer and 4.9 mL Rnase/Dnase-free water. 57.5 µL of this solution was aliquoted to each well of a 96-well plate and 5 µL/well of STRT-FW-n (from 5 µM stock plate) was added, i.e. a different oligo in each well.

The sequence of STRT-T30-BIO (which is a CDS) is:

5'-BIO-AAGCAGTGGTATCAACGCAGAGT$_{30}$VN-3',

and the sequence of STRT-FW-n (which is a TSO) is:

5'-AAGCAGTGGTATCAACGCAGAGTGGATGCTXXXXXrGrGrG-3'(X=cell tag)

BtsCI->2/0

n is 1-96 and each oligonucleotide has a distinct cell tag, such that a different oligonucleotide is added to each well containing a single cell.

Mouse embryonic stem cells (R1) were grown without feeder cells, trypsinized, cleared through cell strainer and resuspended in 1x PBS. Cells were then picked by FACS into the

Capture Plate, with a single cell being placed in each well. The Capture Plate was transferred to a PCR thermocycler and incubated at 72°C for 2 minutes, and then cooled to 4°C for 5 minutes to allow annealing to occur. The detergent in STRT buffer helps reduce adsorption of mRNA and cDNA to the walls of the reaction tube during subsequent steps, and also improves lysis of the cells. The heating step causes the cell to lyse completely and release its RNA. When the temperature is reduced, the oligo(dT) primer anneals.

Reverse transcription

5 µL/well of RT mix (see Table 2 below) was added and the plate was incubated at 42°C for 45 minutes, without heated lid.

Reagent	**For 96 reactions**	**For one reaction**	**Final conc.**
STRT buffer (5x)	110 µL	1 µL	1x
DTT (20 mM)	110 µL	1 µL	2 mM
dNTP (10 mM)	110 µL	1 µL	1 mM
Dnase/Rnase-free water	209 µL	1 µL	
Superscript II RT (200 U/µL)	11 µL	1 µL of 20 U/µL (dilute in 1x RT buffer)	2.5 U/µL
Total volume	**550 µL**	**5 µL**	

Table 2 Composition of RT mix

When the RT mix is added, the reverse transcriptase enzyme (Superscript II RT) synthesizes a first strand and the tagged template-switching oligo introduces an upstream primer sequence.

Figure 2 shows the synthesis of cDNA by template switching. The 5' end of the cDNA (which corresponds to the 3' end of the mRNA) can be controlled by adding a tail (which is oligo-dT in this case) to the cDNA synthesis primer (CDS). The 3' end of the cDNA can be controlled using the template-switching oligo (TSO). When reverse transcriptase reaches the 5' end of the mRNA template, it preferentially adds 2-5 cytosines. The template-switching oligo, which has 2-5 guanosines, anneals transiently, and reverse transcriptase then switches template and synthesizes the complementary strand. By this mechanism, both ends of the cDNA can be arbitrarily controlled.

The structure of a typical TSO is shown in **Figure 3**. In this particular TSO, the 3' template switching sequence includes three riboguanines (rG). The cell-tag is shown as "XXXXX" and

can in general have any length or nucleotide composition. Arbitrary sequence can be inserted at the 5' end of the TSO, after the 5' APS, or after the cell-tag, but not at the 3' end.

The structure of a typical CDS is shown in **Figure 4**. The RCS is oligo-dT with an anchor nucleotide (V = A,C,G degenerate). The cell tag "XXXXX" can have any length or nucleotide composition. Additional arbitrary sequences can be inserted at the 5' end, after the 5' APS, or after the cell-tag.

Purification of cDNA

50 µl PBI (Qiaquick PCR Purification Kit) was added to each well to inactivate reverse transcriptase. The PBI inactivates reverse transcriptase and cDNA from all the wells was then pooled. Adding PBI before pooling prevents cDNA synthesis from proceeding once the cDNA samples have been pooled. The pooled cDNA was loaded on a single Qiaquick column and the purified cDNA was eluted in 30 µl EB buffer into a Beckman Polyallomer tube. The purification step removes primers (<40 bp) as well as proteins and other debris.

Full length cDNA amplification

The cDNA was amplified by PCR by adding the reagents shown in Table 3.

Reagent	**For one tube:**	**Final conc.**
Rnase/Dnase-free water	54 µL	
Advantage2 PCR buffer (10x)	10 µL	1x
dNTP (10 mM)	2 µL	200 µM
STRT-PCR (10 µM)	2 µL	200 µM
Advantage2 DNA Polymerase mix (50x)	2 µL	1x
Total volume	**100 µL**	

Table 3. Reagents used for full-length cDNA amplification.

The sequence of STRT-PCR is:
5'-BIO-AAGCAGTGGTATCAACGCAGAGT-3'

PCR was performed using a heated lid as follows: 1 min @ 95°C, 25 cycles of [5 s @ 95°C, 5 s @ 65°C, 6 min @ 68°C], 4°C forever.

30 µL of the reaction was transferred to a new PCR tube, labeled "Optimization". The remaining 70 µL was stored at 4°C until later. 10 µL from the Optimization tube was removed and the rest of the sample was run for three more cycles. This was repeated to obtain aliquots

from 25, 28 and 31 cycles. A diagnostic 2% agarose gel was used to determine the optimal cycle number (which is the cycle just before saturation of the PCR), as well as to visualize the size range of the product (see **Figure 5**). Typically, the optimal number of cycles was around 28. The remaining 70 µL of reaction was run to reach the optimal number of cycles (in addition to the 25 cycles already run).

The PCR product was purified using a Qiaquick column (PCR purification kit) and eluted in 50 µL EB into a Beckman polyallomer tube. The expected concentration at this stage was about 20-40 ng/µL (1-2 µg total yield).

DNase treatment

The sample was treated with DNaseI in the presence of Mn^{2+} to generate double-strand breaks and reduce the size. First, the following components were mixed in the order shown in Table 4.

Reagent	Volume	Final concentration
cDNA template	50 µl	8 – 16 ng/µL
Water	42.8 µl	
10 x DNase I buffer	11.6 µl	
100 mM $MnCl_2$ (*)	11.6 µl	10 mM
Total volume	**116 µl**	

(*) It is crucial to add $MnCl_2$ last to the reaction, as the BSA present in the buffer will precipitate otherwise.

Table 4. Composition of reaction mix for DNase treatment.

Diluted DNaseI (0.01 units/µl) was prepared just before use as follows: 40 µL 10x DNase I buffer, 318 µL water, 40 µL 100 mM $MnCl_2$ and 2 µL DNaseI (2 U/µL).

4 µl of this diluted DNase I was added to the reaction mix described in Table 4, and was incubated at RT for exactly 10 minutes. The reaction was then stopped by adding 600 µL PBI. The sample was purified on a Qiaquick column and eluted in 30 µL EB.

Bead capture and end repair/nick translation

The fragments were next bound to beads to capture 5' and 3' ends, and then treated with TaqExpress to repair frayed ends and nicks. 30 µL Dynabeads MyOne C1 Streptavidin were washed twice in 2x B&W (Dynal), then added to the Dnase-treated sample, incubated for 10 minutes, and then washed 3x in 1x B&W. About 10% of the sample was bound to the beads (i.e. about 30 – 60 ng), since internal fragments were not biotinylated.

The beads were washed once in 1x TaqExpress buffer and resuspended in the reaction mix shown in Table 5:

Reagent	Volume	Final concentration
10x TaqExpress buffer (blue, with $MgCl_2$)	4 µL	1x
dNTP (10 mM)	0.8 µL	200 µM
Water	33 µl	
TaqExpress (5 U/µL)	2 µL	0.25 U/µL
Total volume	**40 µl**	

Table 5. Composition of the reaction mix used for end repair/nick translation.

The reaction was incubated at 37°C for 30 minutes, and then washed three times in 1x NEB4 buffer.

Fragment release and RDV/FDV adapter ligation

The fragments were released by BtsCI digestion, and simultaneously ligated to the FDV and RDV adapters. The beads were then resuspended in the reaction mix shown in Table 6.

Reagent	Volume	Final concentration
10x NEB4 buffer	4 µL	1x
ATP (10 mM)	4 µL	1 mM
Adapter STRT-RDV-A (10 µM)	4 µL	1 µM
Adapter STRT-FDV (10 µM)	4 µL	1 µM
Water	26 µL	
T4 DNA Ligase (5 U/µL; Invitrogen)	2 µl.	0.25 U/µL
BtsCI (20 U/µL)	2 µl.	1 U/µL
Total volume	**40 µl**	

Table 6. Reaction mix for resuspension of the beads.

The sequence of STRT-FDV, made by annealing STRT-ADP1U and STRT-ADP1L, was:

5`----- CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATCT-3`
3`-PHO-GGTGATGCGGAGGCGAAAGGAGAGATACCCGTCAGCCACTA-PHO-5`

The sequence of STRT-RDV-A, made by annealing STRT-ADP2U-T and STRT-ADP2L was:

5`----- AACTGCCCCGGGTTCCTCATTCTCTT-3`
3`-PHO-TTGACGGGGCCCAAGGAGTAAGAGA-PHO-5`

The beads were incubated for 30 min at 37°C. The reaction was stopped by adding 200 µL PB1, while the beads were held on the magnet. The supernatant was loaded on a Qiaquick

column, purified and eluted in 30 µl EB in a Beckman polyallomer tube. The concentration of the cDNA was about 1 – 2 ng/µL.

Library PCR amplification

Eight reactions were set up using 4, 2, 1, 1/2, 1/4, 1/8, 1/16 and 1/32 µL aliquots of the adapted library, each in 4 µL. Each library was amplified using the PCR reaction mix shown in Table 7.

Reagent	**For one tube:**	**Final conc.**
cDNA library (dilution series)	4 µL	(20 pg/µL)
TaqExpress blue buffer (10x)	5 µL	1x
dNTP (10 mN)	1 µL	200 µM
SOLID-P1 (10 µM)	2 µL	400 nM
SOLID-P2 (10 µM)	2 µL	400 nM
Reagent	**For one tube:**	**Final conc.**
Rnase/Dnase-free water	35.7 µL	0.25 U/µL
TaqExpress polymerase (25 U/µL)	0.3 µL	0.15 U/µL
Total volume	**50 µl**	

Table 7. PCR reaction mix for cDNA library amplification.

The sequence of SOLID-P1 was:

5'-CCACTACGCCTCCGCTTTCCTCTCTATG-3'

The sequence of SOLID-P2 was:

5'-CTGCCCCGGGTTCCTCATTCTCT-3'

PCR was run with heated lid: 5 min @ 94°C, 18 cycles of [15 s @ 94°C, 15 s @ 68°C], 5 min @ 70°C.

All eight reactions were loaded on a 2% E-gel, 10 µL + 10 µL water to determine which reaction was just shy of saturated (see **Figure 6**).

A fresh PCR reaction was then performed using the optimal number of cycles and starting material. For example, if 1/4 µL was optimal at 18 cycles, then 14 cycles were performed.

The PCR product was loaded on a 2% E-gel, 125 – 200 bp region was excised from the gel and purified by Qiagen Gel Extraction Kit (see **Figure 7**). The purified cDNA was eluted in 50 µL EB.

The cDNA library was now prepared for SOLiD sequencing, and could go directly into emulsion PCR.

To verify cDNA library quality, an aliquot was cloned using Invitrogen TOPO TA cloning kit, and sequenced by Sanger sequencing. **Figure 8** shows a typical result demonstrating the presence of primer sequences for SOLiD (P1 and P2; underlined), the cell-specific tag (boxed), and the 2-5 Gs (shaded in a gray box) from the template-switching mechanism. From 22 Sanger sequences, 7 were not mappable to anything in GenBank. All except one of these were misligations of the SOLiD adapters, which can be re-designed to stop this happening. In separate experiments, we found no misligated adapters after blocking their blunt ends with 3' phosphate. Alternatively, the non-ligating 3' ends could be blocked using dideoxy nucleotides or by designing a protruding strand incompatible with the ligating ends of the adapters.

Of the remaining 15 sequences, one was a ribosomal RNA (45S), which is not polyadenylated. It probably occurred due to internal mispriming during first-strand synthesis. The remaining 14 reads were all from polyadenylated mRNA, in the correct orientation and with correct cell tags.

To summarize this dataset, 15 of 22 reads were mappable and 14 of these 15 were correct transcript tags. All the transcripts seen in the Sanger sequence dataset are listed below:

Gene	Length (of mRNA)
Ribosomal protein L35	452
B2_Mm2	~200
Tubulin beta 2c	1 561
B2_Mm1	~195
RIKEN 1110008L16 gene	3 127
Sod2	661
Chchd2	910
mt-Cox2	947
Hnrnpab	2 545
Ribosomal protein L24	558
Ribosomal protein S18	524
RIKEN 2700060E02	941
B2_Mm1	~195
Ribosomal protein S28	356

As expected, the list was dominated by highly expressed genes like ribosomal proteins. Several long transcripts were present in this sample, indicating that there was no strong bias (if any) towards short mRNAs.

Interestingly, three copies of B2 repeats (of subfamilies Mm1 and Mm2) were observed. These are SINE-family repeats expressed from a pol III promoter (not pol II as most mRNAs), but with strong polyadenylation signals. They have been shown to be expressed at extremely high levels in ES cells, together comprising more than 10% of all mRNA. Even more interestingly, they peak just before S-phase in dividing cells, and are thus an early indication that it will be possible to characterize the cell cycle in unsynchronized primary cells using this method.

Quality control by quantitative real-time PCR

To verify that the libraries were representative of the mRNA content of the original ES cell population, quantitative real-time PCR was performed against a set of markers for pluripotency, as well as markers for differentiated tissues. A cDNA library prepared according to classical methods from 1 µg total RNA (~100,000 cells) was compared with the library prepared from 96 single cells using the STRT protocol.

Well-known markers of pluripotency, such as Sox2, Oct4 and Nanog were detected at similar levels in both samples, whereas markers of germ layer differentiation such as Brachyury, Gata4 and Eomes were detected only at very low levels in both samples (see **Figure 9**). The quantitative correlation was good (Pearson correlation coefficient 0.84), with the exception of Plk1, which was not detected in single cells in this experiment.

Reagents used

Reagent	**Source**
2x B&W	10 mM Tris HCl pH 7.5, 1 mM EDTA, 2 M NaCl
10% Tween-20 stock	Make 10% Tween-20, filter at 0.45 µm
Dynabeads MyOne Streptavidin C1	Invitrogen
5x STRT buffer	100 mM Tris-HCl pH 8, 375 mM KCl, 30 mM $MgCl_2$, 0.1% Tween-20
DNase/RNase-free water	Ambion
20 mM DTT	
10 mM and 1 mM dNTP	NEB / In Vitro
Superscript II RT	Invitrogen
10x Advantage2 PCR buffer	Clontech
50x Advantage2 polymerase mix	Clontech
10x DNase I buffer	0.5 M Tris pH 7.5, 0.5 mg/ml BSA
100 mM $MnCl_2$	Sigma 1M stock
Thermo-Fast 96-well plate	AbGene
Thermo-Strip tube strips	AbGene
1.5 mL polyallomer tubes	Beckman-Coulter
2% E-gel with SYBR Safe	Invitrogen

Reagent	Source
Qiaquick PCR Purification Kit	Qiagen
Qiaquick Gel Extraction kit	Qiagen
BtsCI restriction endonuclease	NEB
T4 DNA Ligase	Invitrogen
10 mM ATP	
10x NEB4 buffer	NEB
5 U/μl TaqExpress polymerase	Genetix, UK
10x TaqExpress buffer (blue)	Genetix, UK

Table 8. List of reagents used in the method described above.

EXAMPLE II

Characterization of single-cell transcriptional landscape by highly multiplex RNA-Seq

Understanding of the development and maintenance of tissues has been greatly aided by large-scale gene expression analysis. However, tissues are invariably complex, consisting of multiple cell types in a diversity of molecular states. As a result, expression analysis of a tissue confounds the true expression patterns of its constituent cell types. Described herein is a novel strategy, termed shotgun single-cell expression profiling, was used to access such complex samples. It is a simple and highly multiplexed method used to generate hundreds of single-cell RNA-Seq expression profiles. Cells are then clustered based on their expression profiles, forming a two-dimensional cell map onto which expression data can be projected. The resulting cell map integrates three levels of organization: the whole population of cells, the functionally distinct subpopulations it contains, and the single cells themselves—all without need for known markers to classify cell types. The feasibility of the strategy is demonstrated by analyzing the complete transcriptomes of 436 single cells of three distinct types. This strategy enables the unbiased discovery and analysis of naturally occurring cell types during development, adult physiology and disease.

METHODS

Cell culture

ES R1 cells were cultured as previously described (Moliner et al., Stem Cells Dev. 17:233-243 (2008)). MEFs and Neuro-2A cells were grown in DMEM with 10% FBS, 1x

penicillin/streptomycin, 1x Glutamax and 0.05 mM 2-mercaptoethanol. All culture reagents were from Gibco.

Quantitative real-time PCR (Q-PCR)

RNA was isolated using Trizol (Invitrogen) and 1 µg total RNA was reverse transcribed with Superscript III (Invitrogen) and oligo-(dT) primer. SYBR Green Master Mix (Applied Biosystems) and a cDNA amount corresponding to 5 ng RNA was mixed with 4 pmol primers (Eurofins MWG Operon, Germany) in a total volume of 10 µL, and analyzed on a 7900HT real-time thermocycler (Applied Biosystems). A dilution series of the template was used to determine primer efficiency.

Single-cell tagged reverse transcription (STRT)

Cells were dissociated enzymatically using TrypLE Express (Invitrogen), washed and resuspended in phosphate-buffered saline (PBS). A single cell was collected into each well of a 96-well capture plate (AbGene Thermo-Fast 96 cat. No. 0600) by fluorescence-activated cell sorting (FACS), and the plate was immediately frozen on dry ice. The FACS was used only to collect single cells and to reject dead cells and debris based on light scattering; no fluorescent reporter was used, and hence the collected cells would represent a random sample of the population.

The cell capture plate contained a single cell per well in 5 µL of *STRT* buffer (20 mM Tris-HCl pH 8.0, 75 mM KCl, 6 mM $MgCl^2$, 0.02% Tween-20) with 400 nM STRT-T30-BIO (5'-biotin-AAGCAGTGGTATCAACGCAGAGT$_{30}$VN-3'; this and all other oligos were from Eurofins MWG Operon) and 400 nM STRT-FW-n (5'-AAGCAGTGGTATCAACGCAGAGTGGATGCTXXXXXrGrGrG-3', where "rG" denotes a ribonucleotide guanine and "XXXXX" was a barcode). Each well of the capture plate contained a different template-switching helper oligo (STRT-FW-n) with a distinct barcode. For example, well A01 received STRT-FW-1 with sequence 5'-AAGCAGTGGTATCAACGCAGAGTGGATGCTCAGAArGrGrG-3' having barcode sequence CAGAA. All 96 barcodes and helper oligo sequences are given in Table 9.

Barcode	Oligo name	Sequence (GGG) = ribonucleotides
CAGAA	STRT-FW-1	AAGCAGTGGTATCAACGCAGAGTGGATGCTCAGAA(GGG)
CATAC	STRT-FW-2	AAGCAGTGGTATCAACGCAGAGTGGATGCTCATAC(GGG)
CAAAG	STRT-FW-3	AAGCAGTGGTATCAACGCAGAGTGGATGCTCAAAG(GGG)
CACAT	STRT-FW-4	AAGCAGTGGTATCAACGCAGAGTGGATGCTCACAT(GGG)
CATCA	STRT-FW-5	AAGCAGTGGTATCAACGCAGAGTGGATGCTCATCA(GGG)

Barcode	Oligo name	Sequence (GGG) = ribonucleotides
CAGCC	STRT-FW-6	AAGCAGTGGTATCAACGCAGAGTGGATGCTCAGCC(GGG)
CACCG	STRT-FW-7	AAGCAGTGGTATCAACGCAGAGTGGATGCTCACCG(GGG)
CAACT	STRT-FW-8	AAGCAGTGGTATCAACGCAGAGTGGATGCTCAACT(GGG)
CAAGA	STRT-FW-9	AAGCAGTGGTATCAACGCAGAGTGGATGCTCAAGA(GGG)
CACGC	STRT-FW-10	AAGCAGTGGTATCAACGCAGAGTGGATGCTCACGC(GGG)
CATGT	STRT-FW-11	AAGCAGTGGTATCAACGCAGAGTGGATGCTCATGT(GGG)
CACTA	STRT-FW-12	AAGCAGTGGTATCAACGCAGAGTGGATGCTCACTA(GGG)
CAATC	STRT-FW-13	AAGCAGTGGTATCAACGCAGAGTGGATGCTCAATC(GGG)
CATTG	STRT-FW-14	AAGCAGTGGTATCAACGCAGAGTGGATGCTCATTG(GGG)
CAGTT	STRT-FW-15	AAGCAGTGGTATCAACGCAGAGTGGATGCTCAGTT(GGG)
CCTAA	STRT-FW-16	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCTAA(GGG)
CCGAC	STRT-FW-17	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCGAC(GGG)
CCAAT	STRT-FW-18	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCAAT(GGG)
CCGCA	STRT-FW-19	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCGCA(GGG)
CCTCC	STRT-FW-20	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCTCC(GGG)
CCACG	STRT-FW-21	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCACG(GGG)
CCAGC	STRT-FW-22	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCAGC(GGG)
CCTGG	STRT-FW-23	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCTGG(GGG)
CCGGT	STRT-FW-24	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCGGT(GGG)
CCATA	STRT-FW-25	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCATA(GGG)
CCGTG	STRT-FW-26	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCGTG(GGG)
CCTTT	STRT-FW-27	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCTTT(GGG)
CGAAA	STRT-FW-28	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGAAA(GGG)
CGCAC	STRT-FW-29	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGCAC(GGG)
CGGAG	STRT-FW-30	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGGAG(GGG)
CGTAT	STRT-FW-31	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGTAT(GGG)
CGCCA	STRT-FW-32	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGCCA(GGG)
CGACC	STRT-FW-33	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGACC(GGG)
CGTCG	STRT-FW-34	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGTCG(GGG)
CGGCT	STRT-FW-35	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGGCT(GGG)
CGGGA	STRT-FW-36	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGGGA(GGG)
CGTGC	STRT-FW-37	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGTGC(GGG)
CGAGG	STRT-FW-38	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGAGG(GGG)
CGCGT	STRT-FW-39	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGCGT(GGG)
CGTTA	STRT-FW-40	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGTTA(GGG)
CGGTC	STRT-FW-41	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGGTC(GGG)
CGCTG	STRT-FW-42	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGCTG(GGG)
CGATT	STRT-FW-43	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGATT(GGG)
CTCAA	STRT-FW-44	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTCAA(GGG)
CTAAC	STRT-FW-45	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTAAC(GGG)
CTTAG	STRT-FW-46	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTTAG(GGG)
CTGAT	STRT-FW-47	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTGAT(GGG)
CTACA	STRT-FW-48	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTACA(GGG)
CTGCG	STRT-FW-49	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTGCG(GGG)
CTTCT	STRT-FW-50	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTTCT(GGG)
CTTGA	STRT-FW-51	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTTGA(GGG)
CTGGC	STRT-FW-52	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTGGC(GGG)
CTCGG	STRT-FW-53	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTCGG(GGG)
CTAGT	STRT-FW-54	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTAGT(GGG)

Barcode	Oligo name	Sequence (GGG) = ribonucleotides
CTGTA	STRT-FW-55	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTGTA(GGG)
CTTTC	STRT-FW-56	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTTTC(GGG)
CTATG	STRT-FW-57	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTATG(GGG)
CTCTT	STRT-FW-58	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTCTT(GGG)
GACAA	STRT-FW-59	AAGCAGTGGTATCAACGCAGAGTGGATGCTGACAA(GGG)
GAAAC	STRT-FW-60	AAGCAGTGGTATCAACGCAGAGTGGATGCTGAAAC(GGG)
GATAG	STRT-FW-61	AAGCAGTGGTATCAACGCAGAGTGGATGCTGATAG(GGG)
GAGAT	STRT-FW-62	AAGCAGTGGTATCAACGCAGAGTGGATGCTGAGAT(GGG)
GAACA	STRT-FW-63	AAGCAGTGGTATCAACGCAGAGTGGATGCTGAACA(GGG)
GAGCG	STRT-FW-64	AAGCAGTGGTATCAACGCAGAGTGGATGCTGAGCG(GGG)
GATCT	STRT-FW-65	AAGCAGTGGTATCAACGCAGAGTGGATGCTGATCT(GGG)
GATGA	STRT-FW-66	AAGCAGTGGTATCAACGCAGAGTGGATGCTGATGA(GGG)
GAGGC	STRT-FW-67	AAGCAGTGGTATCAACGCAGAGTGGATGCTGAGGC(GGG)
GACGG	STRT-FW-68	AAGCAGTGGTATCAACGCAGAGTGGATGCTGACGG(GGG)
GAAGT	STRT-FW-69	AAGCAGTGGTATCAACGCAGAGTGGATGCTGAAGT(GGG)
GAGTA	STRT-FW-70	AAGCAGTGGTATCAACGCAGAGTGGATGCTGAGTA(GGG)
GATTC	STRT-FW-71	AAGCAGTGGTATCAACGCAGAGTGGATGCTGATTC(GGG)
GAATG	STRT-FW-72	AAGCAGTGGTATCAACGCAGAGTGGATGCTGAATG(GGG)
GACTT	STRT-FW-73	AAGCAGTGGTATCAACGCAGAGTGGATGCTGACTT(GGG)
GCAAA	STRT-FW-74	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCAAA(GGG)
GCCAC	STRT-FW-75	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCCAC(GGG)
GCGAG	STRT-FW-76	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCGAG(GGG)
GCTAT	STRT-FW-77	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCTAT(GGG)
GCACC	STRT-FW-78	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCACC(GGG)
GCTCG	STRT-FW-79	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCTCG(GGG)
GCGCT	STRT-FW-80	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCGCT(GGG)
GCGGA	STRT-FW-81	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCGGA(GGG)
GCTGC	STRT-FW-82	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCTGC(GGG)
GCAGG	STRT-FW-83	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCAGG(GGG)
GCCGT	STRT-FW-84	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCCGT(GGG)
GCTTA	STRT-FW-85	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCTTA(GGG)
GCGTC	STRT-FW-86	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCGTC(GGG)
GCCTG	STRT-FW-87	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCCTG(GGG)
GCATT	STRT-FW-88	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCATT(GGG)
GGTAA	STRT-FW-89	AAGCAGTGGTATCAACGCAGAGTGGATGCTGGTAA(GGG)
GGCAG	STRT-FW-90	AAGCAGTGGTATCAACGCAGAGTGGATGCTGGCAG(GGG)
GGAAT	STRT-FW-91	AAGCAGTGGTATCAACGCAGAGTGGATGCTGGAAT(GGG)
GGTCC	STRT-FW-92	AAGCAGTGGTATCAACGCAGAGTGGATGCTGGTCC(GGG)
GGACG	STRT-FW-93	AAGCAGTGGTATCAACGCAGAGTGGATGCTGGACG(GGG)
GGCCT	STRT-FW-94	AAGCAGTGGTATCAACGCAGAGTGGATGCTGGCCT(GGG)
GGCGA	STRT-FW-95	AAGCAGTGGTATCAACGCAGAGTGGATGCTGGCGA(GGG)
GGAGC	STRT-FW-96	AAGCAGTGGTATCAACGCAGAGTGGATGCTGGAGC(GGG)

Table 9. Barcodes and helper oligonucleotide sequences.

The cell capture plate was thawed and then heated to lyse the cells (20°C for 5 minutes, 72°C for 4 minutes, 10°C for 5 minutes in a thermocycler). 5 µL reverse transcription mix (4 mM DTT, 2 mM dNTP, 5 U/µL Superscript II in *STRT* buffer) was added to each well and the plate

was incubated (10°C for 10 minutes, 42°C for 45 minutes) to complete reverse transcription and template switching.

To purify the cDNA and remove unreacted primers, 50 µL PB (Qiaquick PCR Purification Kit, Qiagen) was added to each well. All 96 reactions were pooled and purified over a single Qiaquick column. The cDNA was eluted in 30 µL EB in a 1.5 mL polyallomer tube (Beckman).

The whole 96-cell cDNA sample was amplified in a single tube in 100 µL of 200 µM dNTP, 200 µM STRT-PCR primer (5'-biotin-AAGCAGTGGTATCAACGCAGAGT-3'; Eurofins MWG Operon), 1x Advantage2 DNA Polymerase Mix (Clontech) in 1x Advantage2 PCR buffer (Clontech) with 1 min at 94°C followed by 25 cycles of 15 s at 95°C, 30 s at 65°C, 3 min at 68°C, with heated lid. An aliquot was visualized on a 1.2% agarose E-gel (Invitrogen) and the sample was amplified an additional 1-5 cycles if necessary. The product was purified (Qiaquick PCR Purification Kit, Qiagen) and quantified by fluorimetry (Qubit, Invitrogen). Typical yields were 0.5 – 1 µg total. Aliquots were taken at this stage for microarray analysis and Q-PCR.

Sample preparation for high-throughput sequencing

Amplified cDNA was fragmented by DNase I in the presence of Mn^{2+}, which causes a preference for double-strand breaks. 50 µL cDNA was fragmented in DNase I buffer supplemented with 10 mM $MnCl_2$ and DNase I diluted to 0.0003 U/µL in a total volume of 120 µL for exactly six minutes room temperature. The reaction was stopped by the addition of 600 µL PB (Qiaquick PCR Purification Kit, Qiagen), purified and eluted in 30 µL EB into a polyallomer tube (Beckman).

3' and 5' fragments were immobilized on 30 µL streptavidin-coated paramagnetic beads (Dynabeads MyOne C1, Invitrogen), then resuspended in 30 µL TaqExpress buffer (Genetix, UK). Ends were repaired and single A overhangs generated by incubating the beads in 40 µL of 200 µM dNTP, 0.25 U/µL TaqExpress (Genetix, UK) in TaqExpress buffer at 37°C for 30 minutes, followed by three washes in NEBuffer 4 (New England Biolabs).

5' fragments containing barcodes and cDNA inserts were released from the beads by BtsCI digestion, and adapters were simultaneously ligated to generate a sample suitable for sequencing on the Illumina Genome Analyzer. The beads were resuspended in 40 µL of 1 mM ATP, 1 µM SOLEXA-ADP1 adapter

(5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCG ATCT-3' and 3'-PHO-TTACTATGCCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAG GCTA-PHO-5'), 1 µM SOLEXA-ADP2 adapter (5'-CAAGCAGAAGACGGCATACGAGCTCTTCCGATCT-3' and 3'-PHO-GTTCGTCTTCTGCCGTATGCTCGAGAAGGCTAG-PHO-5'), 0.25 U/µL T4 DNA ligase (Invitrogen), 1 U/µL BtsCI (New England Biolabs) in 1x NEBuffer 4, and incubated 30 minutes at 37°C. The beads were removed and the supernatant was purified using AmPure (Agencourt) and eluted in 40 µL EB (Qiagen).

The sample was loaded on a 2% SizeSelect E-gel and the range 200 – 300 bp was collected. A 4 µL aliquot was amplified in 50 µL total volume containing 200 µM dNTP, 400 nM each primer (5'- AATGATACGGCGACCACCGA-3' and 5'-CAAGCAGAAGACGGCATACGAG-3') and 0.15 U/µL Phusion polymerase in Phusion HF buffer (New England Biolabs) with 30 s at 98°C, 14-18 cycles of [10 s at 98°C, 30 s at 65°C, 30 s at 72°C] followed by 5 min at 70°C. Test amplifications were used to determine the minimal number of cycles needed. The amplified sample was purified by Qiaquick PCR Purification followed by a 2% SizeSelect E-gel, again collecting the region 200 – 300 bp. The concentration was measured by Qubit (Invitrogen) and was typically 5 ng/µL. Aliquots were cloned (TOPO, Invitrogen) and sequenced by Sanger sequencing to verify sample quality and determine the average fragment length. Based on this information, the molar concentration could be accurately determined and was generally above 10 nM. Cluster formation and sequencing-by-synthesis was performed on a Genome Analyzer *IIx* according to the manufacturer's protocols (Illumina, Inc., San Diego, USA) at a commercial service provider (Fasteris SA, Geneva, Switzerland).

Mapping, quantification and visualization

Raw reads were sorted by barcode (first five bases) and trimmed to remove up to five 5' Gs introduced by template-switching, and 3' As that sometimes occurred when a read extended into the poly(A) tail. Only exact barcodes were allowed, and the barcodes were designed so that no single error would convert one valid barcode into another. The reads were then mapped to the mouse genome using Bowtie (Langmead et al., Genome Biol. 10:R25 (2009)) with the default settings. Unmapped reads were discarded. Then, for each annotated feature in the NCBI 37.1 assembly, all mapping reads were counted to generate a raw count. That is, all reads that mapped to any of the exons of a gene were assigned to that gene; isoforms were not

distinguished. Finally, the raw reads for each cell were normalized to transcripts per million (t.p.m.). Wells with fewer than 1000 mapped reads were omitted from further analysis; presumably these included cases where the FACS instrument had failed to hit the reagent droplet while cells were picked.

To visualize cells in a two-dimensional landscape, all pairwise similarities were first computed. The Bray-Curtis distance was used as a similarity metric because it tended to handle the noise in low-expressed genes well. Standard correlation yielded similar results, but with a few more misplaced cells (data not shown). A similarity graph was then built by letting nodes represent cells, and connecting each cell to its five most similar cells (for clarity, cells with fewer than 10,000 reads were omitted, as they were liable to generate spurious edges). Thus every node (cell) had five outgoing edges and varying numbers of incoming edges. Force-directed layout was then used to project the graph to two dimensions, revealing the internal structure based on cell-cell similarities. The GraphPlot function of the Mathematica program (Wolfram Research Inc., USA) was used with the "SpringElectricalEmbedding" option.

RESULTS

Data from 436 single cells collected from three different mouse cell types: embryonic stem cells (ES R1, Wood et al., Nature 365: 87-89 (1993)), a neuroblastoma tumor cell line (Neuro-2A, Olmsted et al., Proc. Natl. Acad. Sci U.S.A. 65:129-136 (1970)) and embryonic fibroblasts (MEF) are reported. In brief, each sample was prepared by picking single cells by fluorescence-activated cell sorting (FACS) into the wells of a 96-well PCR plate preloaded with lysis buffer; heating the plate to complete lysis, then adding reverse transcription reagents to generate a first strand cDNA. To incorporate a well-specific (and hence cell-specific) barcode, the reverse transcriptase template-switching mechanism (Schmidt et al., Nucleic Acids Res. 27:e31 (1999)) was used whereby a helper oligo directs the incorporation of a specific sequence at the 3' end of the cDNA molecule (**Figure 10A**). A different helper oligo was used in each well, with distinct five-base barcodes and universal primer sequence. After cDNA synthesis, the 96 reactions were pooled, purified and amplified by single-primer PCR in a single tube. Cell-to-cell amplification bias was thus reduced, and the number of PCR cycles could be kept low since amplification started from 96-fold more material. The amplified samples were then adapted for sequencing using standard methods. The procedure was named "STRT" (single-cell tagged reverse transcription). For details, see the Methods section and **Figure 11**.

5 – 12 million raw reads per sequencing lane were typically obtained on an Illumina Genome Analyzer IIx, and each sample was analyzed on up to eight lanes (but typically one or two). Reads lacking a proper barcode, mostly caused by errors in sample preparation such as misligated adaptors, were removed. Of the remaining 79±11% (mean±s.d.) reads, about three-quarters (75±12%) could be placed on the mouse genome allowing for up to two sequencing errors, resulting in hits to 14,718±3,006 distinct features (including mRNA, mitochondrial RNA and expressed repeats). These results are summarized in Table 10

Library	*Species*	*Cell source*	*Lanes*	*Length (bp)*	*Raw reads*	*Barcoded*	*Mapped*	*Features*	Cum. features
L006	Mouse	ES R1	8	1x36 7x50	46,130,389	38,284,613 (83%)	21,356,066 (56%)	13,168	13,168
L013	Mouse	ES R1	1	36	6,482,712	5,328,081 (82%)	3,199,050 (60%)	19,328	21,285
L019A	Mouse	ES R1	1	36	4,302,649	2,588,536 (60%)	2,344,394 (91%)	12,229	
L019B	Mouse	ES R1 (*)	1	36	5,134,171	3,323,243 (65%)	2,562,559 (77%)	14,794 (**)	23,152
L023	Mouse	Neuro2A	1	50	10,062,086	8,588,048 (86%)	5,963,143 (69%)	11,464	23,735
L026A	Mouse	MEF	1	36	4,278,888	3,729,229 (87%)	2,862,951 (77%)	12,924	
L026B	Mouse	MEF (*)	1	36	3,912,144	3,398,519 (87%)	2,599,074 (76%)	13,832 (**)	24,288

(*) these samples were independently amplified from corresponding 'A' libraries, but were not quality-controlled by Sanger sequencing at preparation

(**) cumulative with the previous sample (i.e. A and B together)

Table 10. Library summaries. Percentages refer to percent of the preceding column unless otherwise noted. "Features" includes mRNA, mitochondrial RNA and expressed retrotransposon families. "Cumulative features" tracks the cumulative number of features across all lib

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Hits spanned some transcripts (**Figure 10B**), but were more commonly located in a region approximately 200 – 1500 bp from the 3' end of each gene, as illustrated in **Figure 10C**. This was expected because an oligo(dT) primer was used to generate cDNA from the 3' end and the mRNA was likely partially degraded during cell lysis by high-temperature hydrolysis in the presence of Mg^{2+}. The background from e.g. genomic DNA contamination, judged by hits to unannotated sequence, was minimal ($<10^{-3}$ reads per million per kilobase), as clearly seen in **Figure 10B** and **Figure 10C**. Note the paucity of hits to the reverse strand and to introns for both Pou5f1 and Nanog; other loci were similar.

Scrutinizing the mapped data, no evidence of mispriming or other undesired side-reactions was found. Control experiments showed that both RNA, reverse transcriptase and template switching oligo were individually required to yield product (data not shown). The vast majority of mapped reads had a properly oriented barcode, indicating that they were primed from the oligo-dT primer and correctly template-switched. No evidence of a motif complementary to any of the primers near read mapping sites or indeed of any other motif was found, except for a weak general T-bias in rare cases (**Figure 12**). On the other hand, there were frequent hotspots of template switching (**Figure 13**), indicating structural constraints in RNA affecting cDNA synthesis and/or template-switching at particular sites. Hotspots were consistent between cells, and should therefore not affect quantitative comparisons.

To characterize sample complexity, and to determine the depth of sequencing required to sample most of the available complexity, the 'new discovery' rate as function of read depth was studied. In other words, the number of new, distinct molecules that were discovered as more sequences were added was determined. It should be noted that, at most, one amplifiable clone was generated from each polyadenylated RNA molecule and this clone was then amplified and sequenced from its 5' end. Therefore reads mapping to distinct locations must have been generated from distinct mRNA molecules. On the other hand, reads mapping to the same location may have been coincidentally generated from two mRNA molecules, or may represent copies of the sample initial clone. The number of distinctly mapping reads was therefore a lower bound on the true sample complexity. As shown in **Figure 14A**, none of the samples reported here were sequenced to saturation, even at 21 million mapped reads, but the yield of new molecules tended to slow down after about 5-10 million reads. Projecting the curves to infinite read depth, most samples would seem to contain at least three million distinct molecules, or about 30,000 per cell. Assuming $10^5 - 10^6$ mRNA molecules per cell, this would suggest the method successfully converted 3-30% of the mRNA to mappable reads, as a (very)

conservative lower bound. The true proportion was likely significantly higher, as many coincident reads (discarded in this analysis) would be generated from template switching hot spots, as mentioned above.

In contrast, the rate of discovery of distinct features rapidly diminished, and 86% of all distinct feature were detected in the first 14% reads (**Figure 14B**). This suggests that the method successfully recovered most expressed features present in the samples, even at relatively low read depth.

Strand information is often required to properly assign reads to transcriptional units, since genes frequently overlap on opposite strands. For example, more than 3000 human genes overlap in this manner (Yelin et al., Nat. Biotechnol. 21:379-386 (2003)). Because the template-switching mechanism used to introduce a barcode occurs directionally, strandedness could be preserved throughout the protocol. To confirm this, the mitochondrial genome was examined, which is expressed as a single long transcript from one strand (the H strand), and is subsequently cleaved to excise tRNA transcripts located between protein-coding genes. Only the protein-coding genes are then polyadenylated. A single protein-coding transcript, ND6, is generated from the L strand, but it is very weakly expressed and irregularly polyadenylated (Slomovic et al., Mol. Cell. Biol. 25:6427-6435 (2005)). As shown in **Figure 10D**, very strong strand-specificity (>99% of reads on the H strand) was observed and no significant expression of tRNA genes was detected, which confirms that the method was poly(A)-specific. The small number of hits on the L strand occurred mainly near the L strand promoter, which may be explained by the polyadenylation of aborted L-strand transcripts (Slomovic et al., *supra*). Similarly, the apparent expression of ND6 on the wrong strand is likely explained by the natural polyadenylation of ND5 downstream of its open reading frame (Slomovic et al., *supra*). The strand specificity allowed us to unambiguously assign reads to expressed transcripts, even in cases where two overlapping genes were co-expressed (**Figure 15**).

On the larger scale of the nuclear chromosomes, hits were approximately equally distributed on the forward and reverse strands. Read density correlated strongly with gene density as shown for chromosome 19 in **Figure 10E**, again indicating that most reads originated specifically from expressed transcripts and were accurately mapped to the genome.

In order to generate a quantitative measure of gene expression, the number of hits to each annotated feature, normalized to transcripts per million (t.p.m.) were counted. Assuming 10^5 to 10^6 transcripts per cell, 1 to 10 t.p.m. corresponds to a single mRNA molecule per cell.

Transcript length (as in the RPKM measure (Mortazavi et al., Nat. Methods 5:621-628 (2008))) was not used to normalize because a single amplifiable 3'-end molecule was generated for each input mRNA molecule, irrespective of its length. An advantage of this approach was the lack of bias against short transcripts (which must be sampled more deeply to generate a detectable RPKM value) or long transcripts (which might otherwise be suppressed during PCR). Indeed, and in contrast to standard RNA-Seq (Oshlack et al., Biol. Direct 4:14 (2009)), no length-dependent bias for transcripts longer than 800 nucleotides was observed (**Figure 16**). Transcripts shorter than about 200 nucleotides were undercalled, likely because only samples above 100 bp were gel-selected. Additionally, transcripts around 600 nucleotides were slightly overrepresented, possibly because of the higher efficiency of template-switching at the 5'-cap of mRNA (Schmidt et al., Nucleic Acids Res. 27:e31 (1999)) or due to the presence of a few very highly expressed genes in this range (e.g. Dppa5 and Rps14).

Expression levels spanned four orders of magnitude in single cells (approximately 1 – 10,000 t.p.m.), with most genes expressed at low levels (<100 t.p.m.: **Figure 17A**). Given the relatively shallow depth of sequencing used here, genes expressed below 10 t.p.m. were generally undetectable solely due to the sampling limit. Since single-cell cDNA was pooled before amplification, the yields of different cells could not be subsequently normalized. As a consequence, cells were unequally sampled and the limit of detection varied. For example, compare two cells sampled at 500,000 reads (**Figure 17B**) and 100,000 reads (**Figure 17C**). In the former case, the apparent detection limit was around 10 t.p.m., whereas in the latter case genes below 100 t.p.m. were generally not detected. However, in both cases, genes above the detection limit were reproducibly quantified in single cells (the coefficient of variation was 46% at 500,000 reads; and 72% at 100,000 reads). Extending this analysis to all cells and genes showed that sensitivity approached the theoretical limit imposed by sampling depth (**Figure 17D**); the difference can be explained by losses in reverse transcription, template switching and sample handling. Measured expression levels were generally accurate, as determined by comparison with Q-PCR (**Figure 17E**), and microarray hybridization (**Figure 18**). In agreement with published reports based on Q-PCR (Bengtsson et al., Genome Res. 15:1388-1392 (2005)), Actb mRNA abundance showed an approximately log-normal distribution across cells (**Figure 19**). RNA polymerase II (large subunit) was expressed at 25±123 t.p.m. in ES cells, comparable to the 27 RPKM found by RNA-Seq (Cloonan et al., Nat. Methods 5:613-619 (2008)) and to the 33±79 t.p.m. found in CHO cells by direct detection *in situ* (assuming 300,000 transcripts per cell) (Raj et al., PLoS Biol 4:309 (2006)).

The cell-cell relationships on a two-dimensional map were visualized, such that more closely related cells would be located near each other. In this way, cell types based solely on expression data were able to be detected and distinguished, without relying on pre-existing markers. A conventional principal component analysis (PCA) revealed three distinct groups of cells, as expected (**Figure 20**). However, a more complete separation into distinct cell-type clusters was achieved using a graph-based method (see **Methods**). Briefly, a graph with nodes representing cells, and edges representing cell-cell similarity of expression pattern was constructed (**Figure 21A**). Force-directed layout to project the graph to two dimensions was used. In this test case using only two cell types (ES and Neuro2A cells), a near-perfect separation was achieved (**Figure 21B**). A larger map incorporating MEFs and additional ES cells showed good separation (**Figure 21C**): demonstrating that the single-cell expression profiles contained enough information to distinguish cell types *de novo*. Both PCA and graph-based analysis clearly distinguished the cell types tested here, but the graph-based method generated more homogenous, well-separated clusters. Both methods accurately grouped independently prepared ES cells together as distinct from the other cell types, showing that clusters did not represent sample preparation artifacts.

Gene expression data was projected onto the map, which provided an easy way to quickly grasp gene expression patterns in both single cells and in the clusters representing cell types (**Figure 22**). A set of well-known ES cell markers (Dppa5, Sox2, Sall4, Pou5f1, Nanog, Zfp42, Zic3, Esrrb) were clearly specifically expressed in ES cells, although their expression levels varied widely from cell to cell (note the logarithmic color scale). A few genes important for pluripotency (Klf4, Myc and Klf2) were more broadly expressed. The power of large-scale single-cell analysis was evident in the fact that while not every cell expressed every marker, patterns of gene activity were highly consistent at the cluster level. For example, even a highly expressed cytoskeletal gene like Actb was not always detected, but its expression in each of the three major clusters was obvious. Correspondingly, lower expressed transcription factors characteristic of ES cells went undetected in some individual ES cells, but the overall pattern of expression in the ES cell cluster was unambiguous and consistent with their identity as ES cells. In general, as the average expression levels decreased from 45 000 t.p.m. (mt_Rnr2) to 1700 (Actb), 850 (Rpl4), 73 (Kras) and 0 t.p.m. (Calb1), the number of expressing cells also decreased, reflecting the stochastic nature of gene expression as well as the sensitivity limits of the method.

The cell map representation demonstrated that (1) individual cells showed highly variable expression patterns, yet their overall pattern of expression was sufficient to group cells of one type together as a cluster; (2) once a cluster of cells was formed, representing a distinct cell type, patterns of gene expression (at the cluster level) were unambiguous. Thus, shotgun single-cell expression profiling is an efficient strategy to access single-cell expression data in heterogeneous populations of cells.

DISCUSSION

Described herein is a reliable and accurate method to obtain RNA-Seq transcription profiles from hundreds of single cells, and shown that single-cell expression profiles can be used to form cell type-specific clusters. This allows analysis of cell type-specific patterns of gene expression both at the single-cell level and the population level, without the need for known markers or even a prior knowledge that a certain cell type exists. This general strategy can be extended to study all kinds of mixed samples. For example, it could be applied to monitor the emergence of specific cell types during organogenesis, without the need to purify those cell types using cell surface markers. Similarly, it could be used to study small populations of stem cells embedded in adult tissues, such as the stem cells that maintain intestinal crypts. The method could also be applied to disease, including the characterization of heterogeneous tumor cell samples or the rare circulating cancer cells that can contribute to metastasis.

What unites all these disparate scientific lines of inquiry is the need to unmix heterogeneous populations of cells. Currently, unmixing is primarily achieved either by physically isolating cells based on known cell surface markers, or by genetically labeling the desired cells so that they can be isolated based on e.g. GFP expression. However, the use of previously known markers precludes the discovery of new cell types, and always risks resulting in mixed data if the markers were not truly specific. In contrast, the methods described herein have shown that cells of distinct types can be unmixed purely *in silico*, provided that large numbers of single-cell expression profiles are generated.

Importantly, then, a very high-throughput, scalable method for single-cell expression profiling is required. Therefore, a method was developed to prepare a barcoded single-cell cDNA sample from 96 cells in a single incubation step. As a consequence, 96 cells could be pooled and treated as a single sample throughout the procedure, which greatly increased throughput and reduced cost. It can also reduce amplification bias, since all 96 cells were amplified in a single closed tube. The entire procedure takes two days to perform, from 96 living cells to

finished samples loaded on the Genome Analyzer. The cost, including all reagents and consumables to generate 10-15 million 36 bp reads using commercial services, was approximately $3500 (that is, about $35/cell).

The date generated herein was on a large number of single cells, each analyzed at a relatively shallow depth of coverage. This allowed the generation of data on far more single cells than have ever been reported in a single study (no single-cell transcriptome experiments with more than a dozen cells have been published), and to produce a cell map with high resolution. In fact, provided that each cell is sampled deeply enough to cluster correctly, it would often make more sense to analyze a larger number of cells than to analyze each cell more deeply. The more cells are added, the more accurate will be the aggregate data obtained from each distinct cell type (cluster), and the better the resolution in "cell type space". For example, many of the ES cells here were sampled at less than 100,000 reads/cell, but altogether 160 ES cells were identified in the cell map, comprising over 1.5 million reads. Sampling a large number of cells will be especially important when the approach is applied to complex tissues, where some types of cells can be present only in a small minority. In addition, as sequencing costs continue to decrease, the tradeoff between number of cells and number of reads will become less pressing.

The use of very large-scale single-cell transcriptional profiling to build a detailed map of naturally occurring cell types is envisioned, which would give unprecedented access to the genetic machinery active in each type of cell at each stage of development. The same strategy can be used to dissect the mutational heterogeneity of neoplasms at the single-cell level.

Throughout this application various publications have been referenced. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains. Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention.

What is claimed is:

1. A method of preparing a cDNA library from a plurality of single cells, the method comprising the steps of:

(i) releasing mRNA from each single cell to provide a plurality of individual mRNA samples, wherein the mRNA in each individual mRNA sample is from a single cell;

(ii) synthesizing a first strand of cDNA from the mRNA in each individual mRNA sample and incorporating a tag into the cDNA to provide a plurality of tagged cDNA samples, wherein the cDNA in each tagged cDNA sample is complementary to mRNA from a single cell;

(iii) pooling the tagged cDNA samples; and

(iv) amplifying the pooled cDNA samples to generate a cDNA library comprising double-stranded cDNA.

2. The method according to claim 1, wherein in step (ii) the tag is incorporated into the cDNA during its synthesis.

3. The method according to claim 1 or 2, wherein synthesis of the first strand of cDNA in step (ii) is directed by a cDNA synthesis primer (CDS) that includes an RNA complementary sequence (RCS) that is at least partially complementary to one or more mRNA in an individual mRNA sample.

4. The method according to claim 3, wherein the RCS is at least partially complementary to a portion of the first strand of cDNA, such that it is able to direct the synthesis of a second strand of cDNA using the first strand of cDNA as a template.

5. The method according to claim 3, wherein a template-switching oligonucleotide (TSO) is added to each individual mRNA sample, wherein said TSO comprises a portion which is at least partially complementary to a portion at the 3' end of the first strand of cDNA.

6. The method according to claim 1, wherein the first strand of cDNA includes a 3' portion comprising a plurality of cytosines or cytosine analogues that base pair with guanosine.

7. The method according to claim 6, wherein the TSO includes a 3' portion comprising a plurality of guanosines or guanosine analogues that base pair with cytosine.

8. The method according to claim 7, wherein the guanosines are ribonucleosides or locked nucleic acid monomers.

9. The method according to claim 5, wherein the CDS or the TSO includes a tag.

10. The method according to claim 5, wherein both the CDS and the TSO include a tag.

11. The method according to claim 10, wherein the CDS and the TSO each include a different tag, such that the tagged cDNA sample comprises a combination of tags.

12. The method according to claim 9, wherein the tag is a nucleotide sequence of 4-20 nucleotides in length.

13. The method according to claim 1, wherein each cDNA sample has a distinct tag or combination of tags.

14. The method according to claim 3, wherein the CDS comprises a 5' amplification primer sequence (APS) and a 3' RCS.

15. The method according to claim 14, wherein the 3' RCS comprises oligo(dT), a gene family-specific sequence, a random sequence or a non-self-complementary semi-random sequence.

16. The method according to claim 14, wherein the TSO includes a 5' APS.

17. The method according to claim 16, wherein the CDS and the 5' APS of the TSO is at least 80% identical to the 5' APS of the CDS.

18. The method according to claim 16, wherein the CDS and the 5' APS of the TSO is 100% identical to the 5' APS of the CDS.

19. The method according to claim 1, wherein the cells are lysed to release mRNA.

20. The method according to claim 1, wherein the mRNA is purified following step (i).

21. The method according to claim 1, wherein the synthesis of cDNA from mRNA is stopped before the tagged cDNA samples are pooled.

22. The method according to claim 1, wherein the tagged cDNA samples are purified before amplification of the cDNA.

23. The method according to claim 1, wherein in step (iv) the pooled cDNA samples are amplified by PCR.

24. The method according to claim 23, wherein the pooled cDNA samples are amplified by emulsion PCR.

25. The method according to claim 23 or 24, wherein the pooled cDNA samples are amplified by single primer PCR.

26. The method according to claim 1, wherein the method further comprises processing the cDNA library to obtain a library suitable for sequencing.

27. The method according to claim 26, wherein the processing comprises fragmenting the cDNA library.

28. The method according to claim 26 or 27, wherein the processing includes the step of adding an adapter to the cDNA.

29. The method according to claim 26, wherein the cDNA library is amplified.

30. A cDNA library produced by the method of claim 1 or 26.

31. A method for analysing gene expression in a plurality of single cells, the method comprising the steps of:

(i) preparing a cDNA library according to the method of claim 1 or 26; and

(ii) sequencing the cDNA library.

32. The method according to claim 31, wherein sequencing is by shotgun sequencing.

33. The method according to claim 32, wherein the cDNA library is sequenced to obtain at least 10,000, at least 1 million, at least 10 million, at least 100 million, or at least 1 billion reads, wherein a read is a length of continuous nucleic acid obtained by a sequencing reaction.

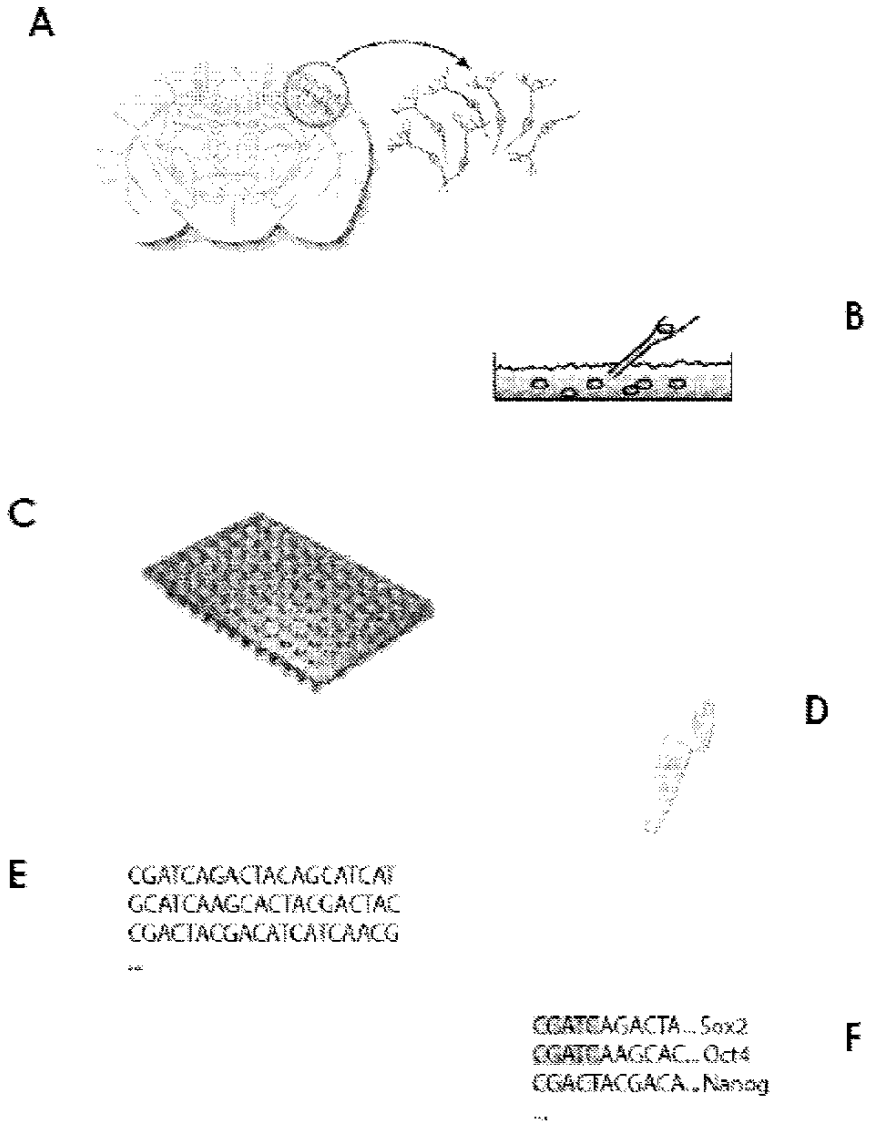


Figure 1

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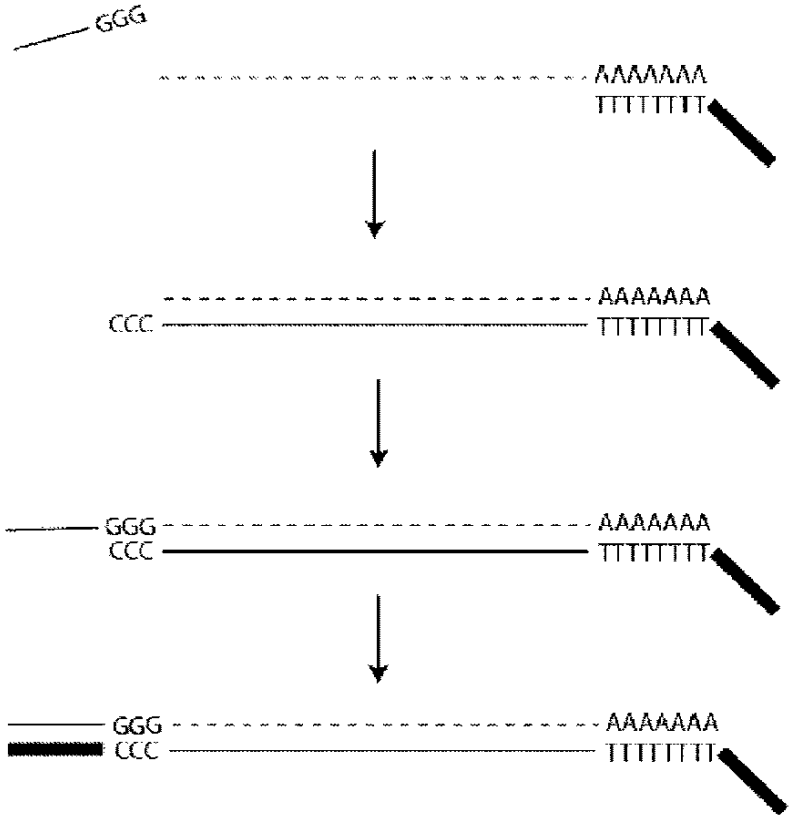


Figure 2

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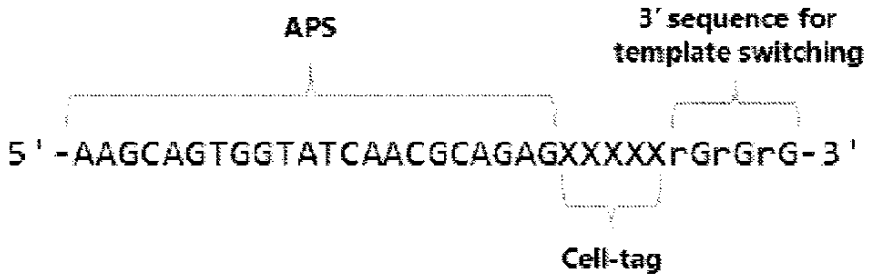


Figure 3

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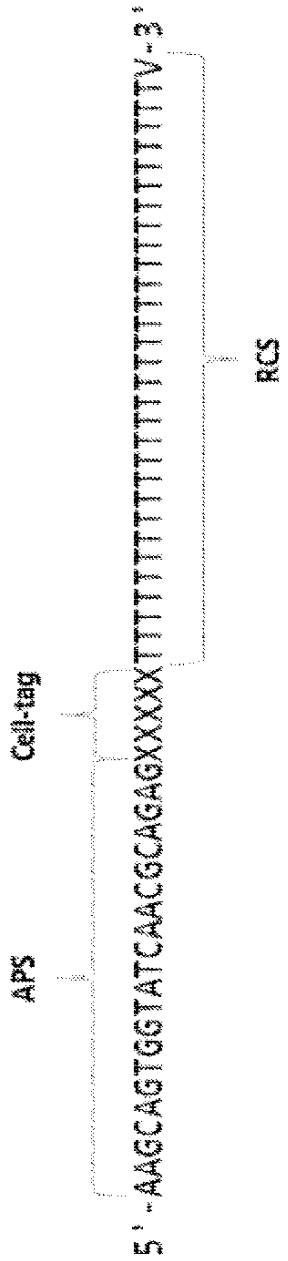


Figure 4

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01016

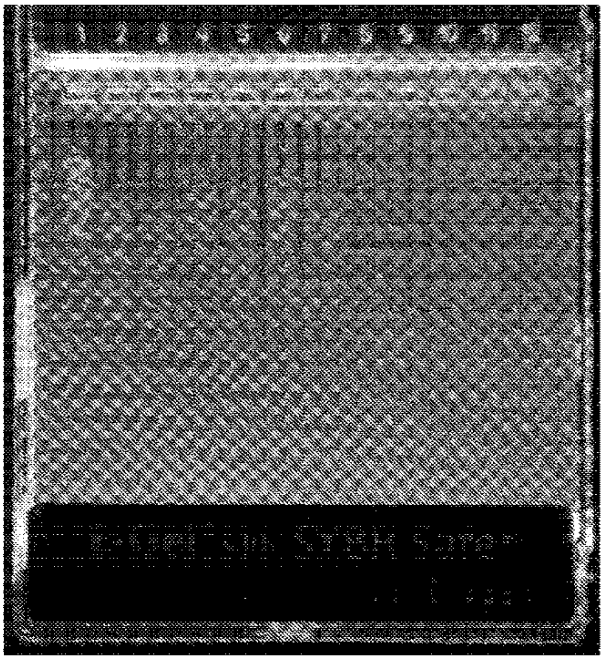


Figure 5

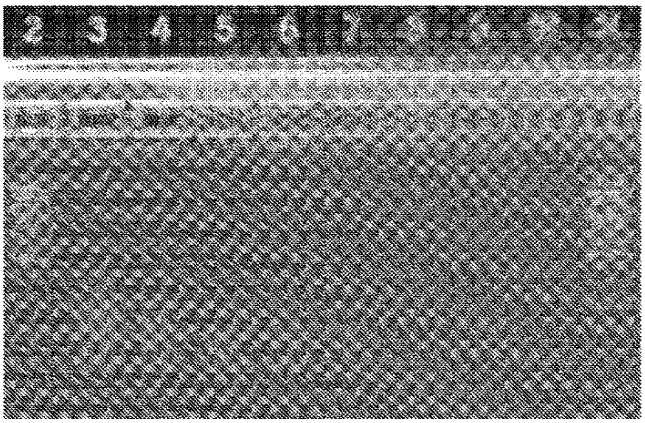


Figure 6

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01017

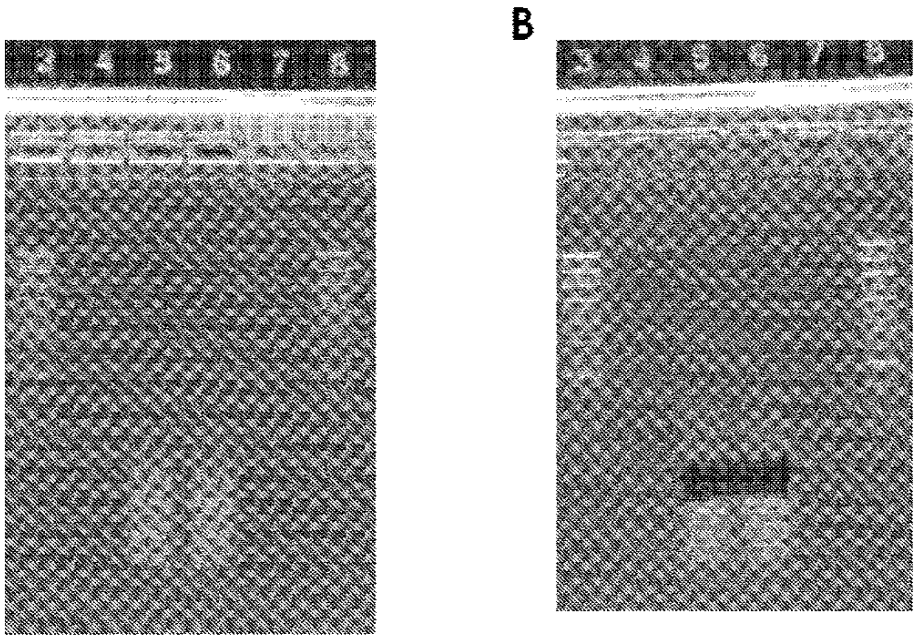


Figure 7

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01018

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GCCTCCGCTTTC
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CGTGTTCAGGGGC
CGCATGAAGAGAATGAGGAACCCGGGGCAG*AAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGT*
GGATCCGAGCTCG
GTACCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCC
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Figure 8

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 01019

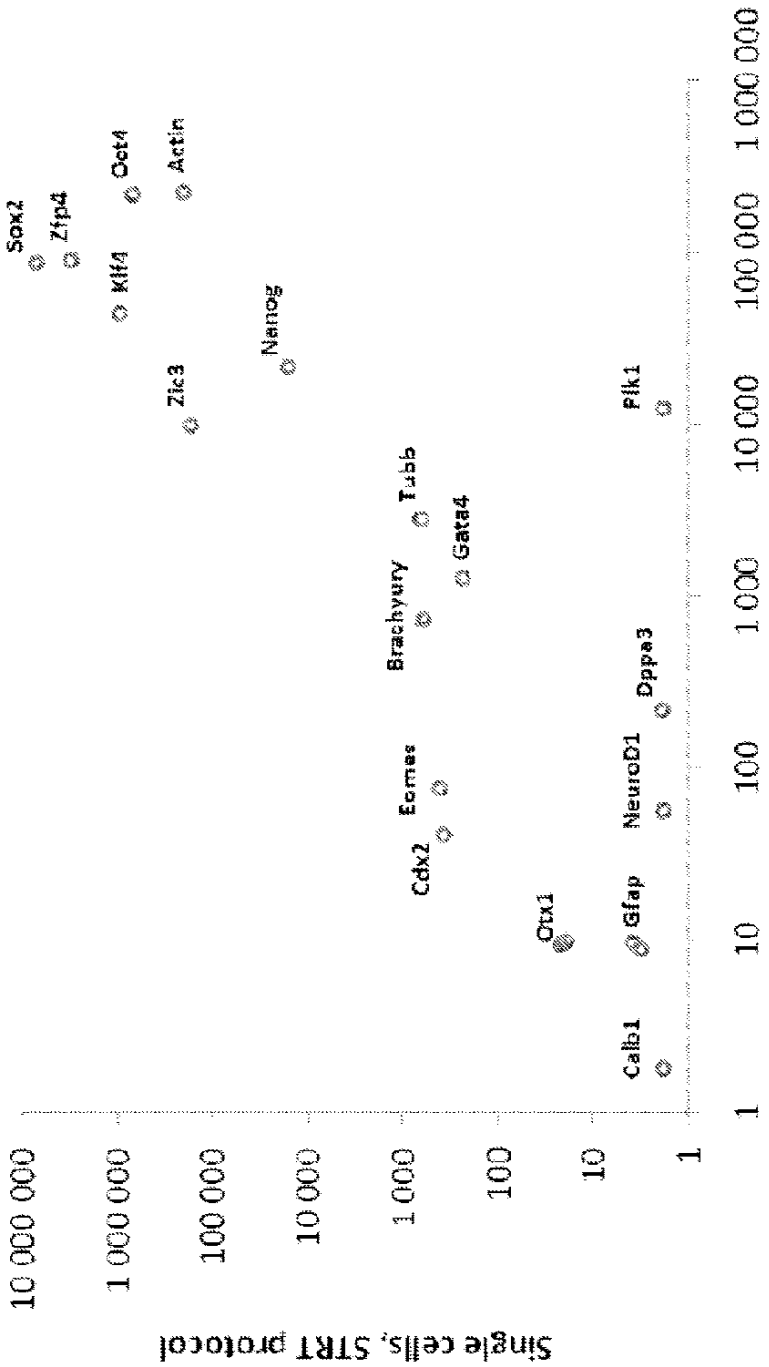


Figure 9

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01020

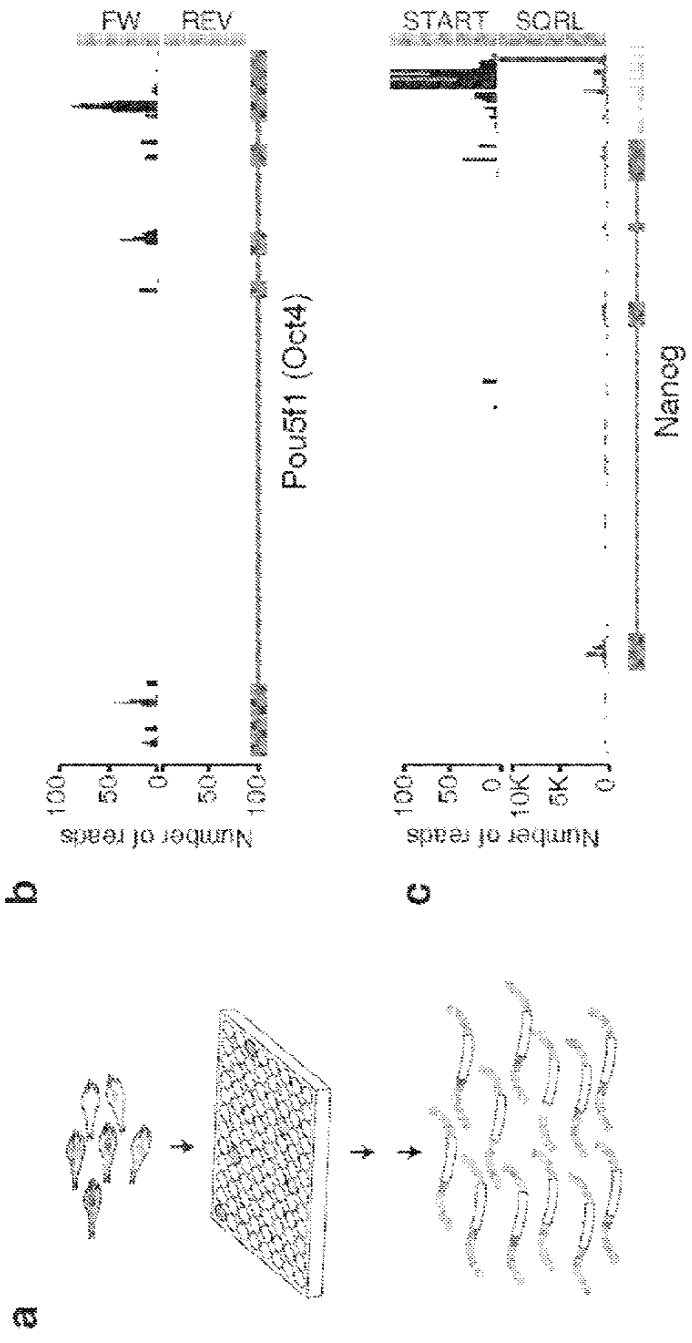


Figure 10A-C

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01021

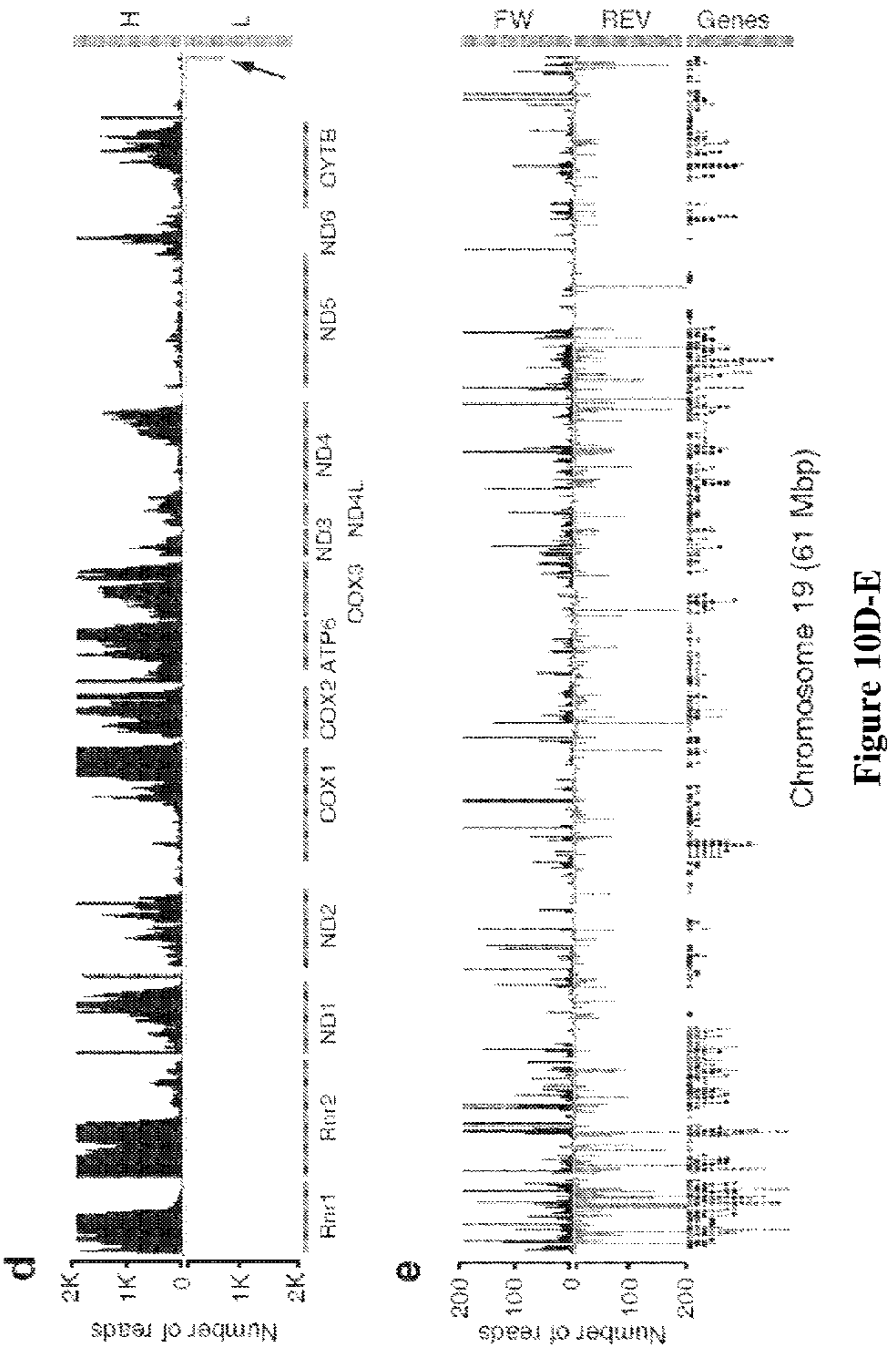


Figure 10D-E

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01022

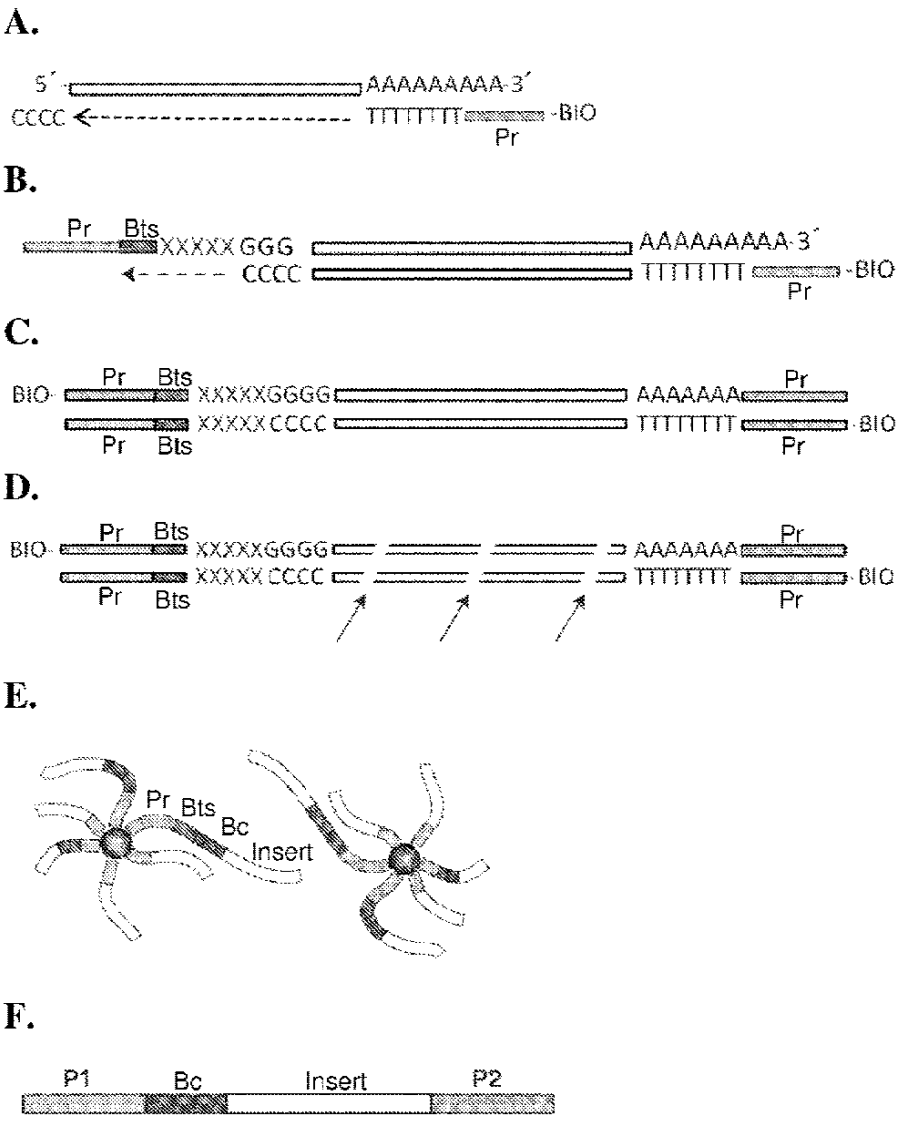


Figure 11

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01023

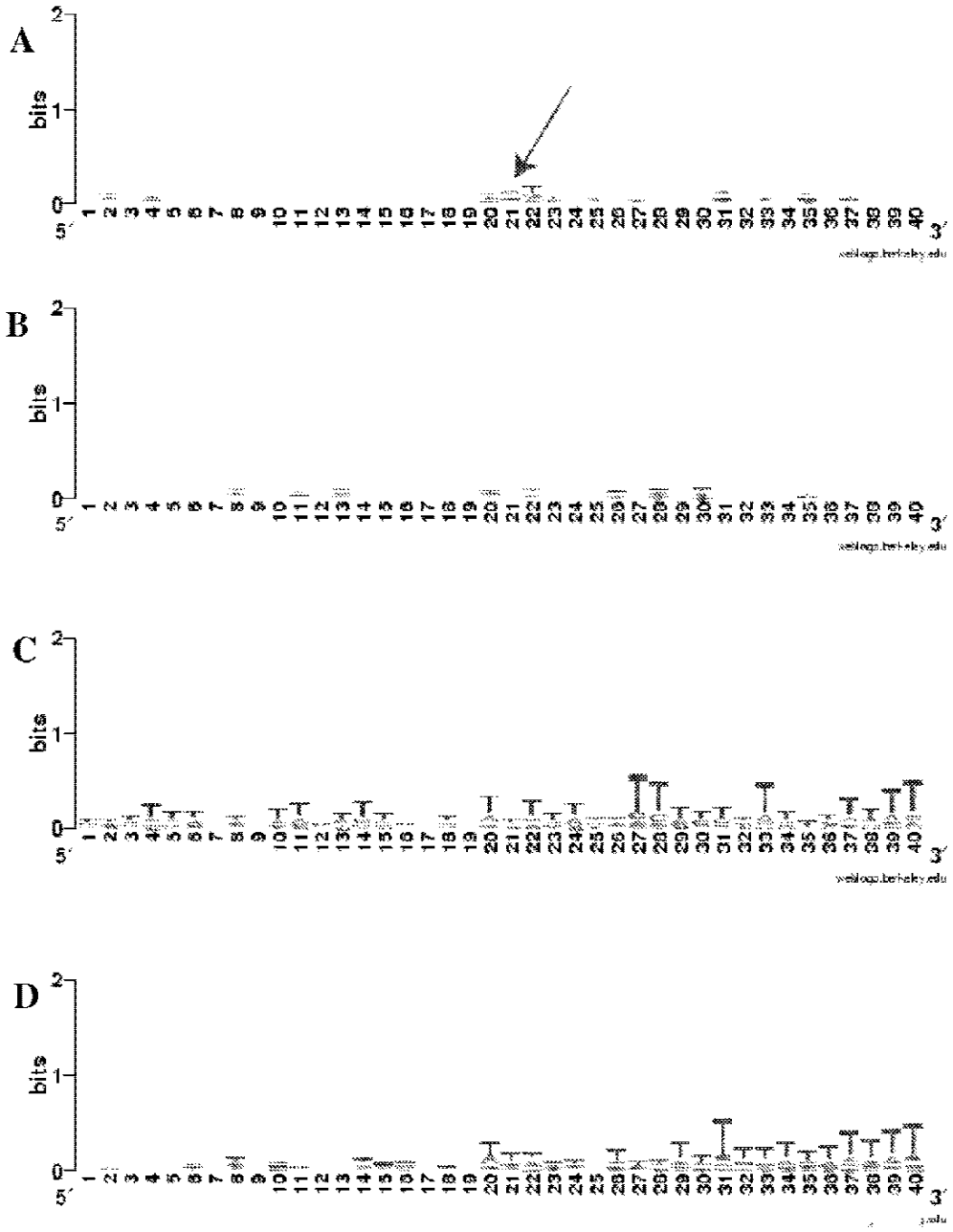


Figure 12

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01024

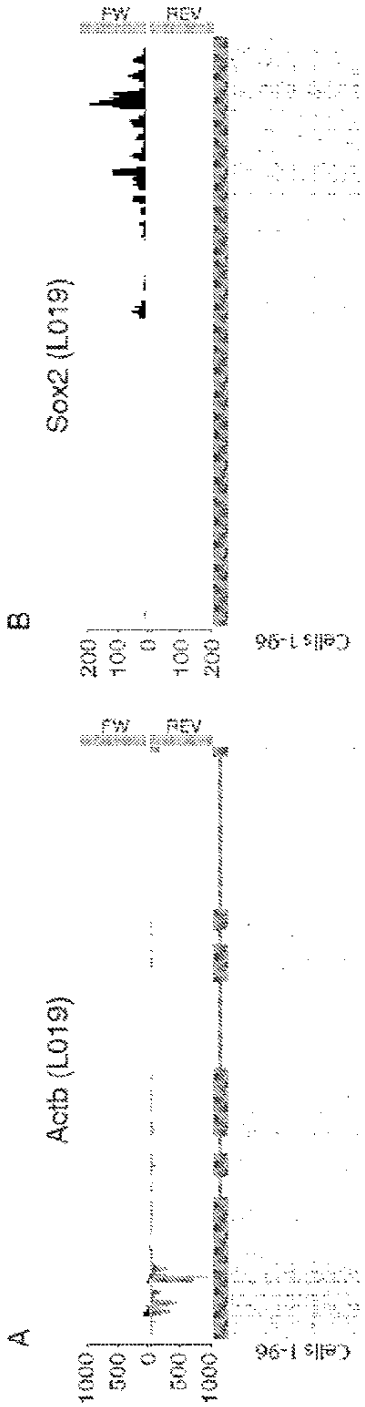


Figure 13

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01025

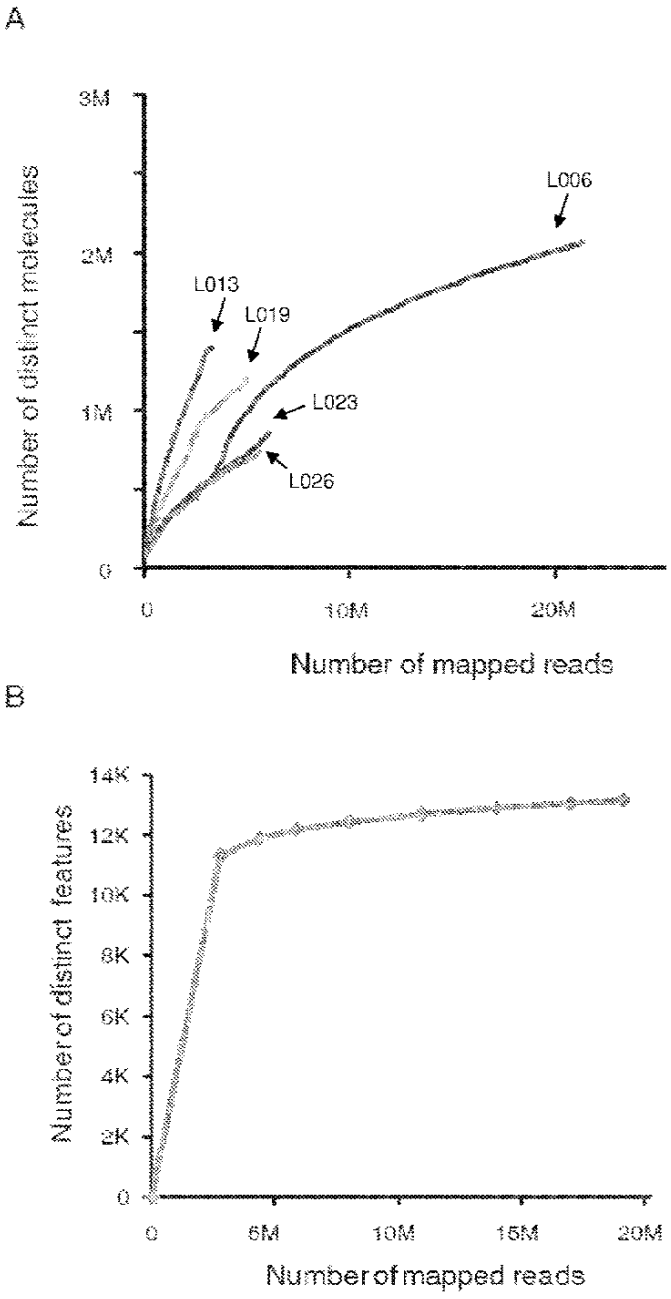


Figure 14

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01026

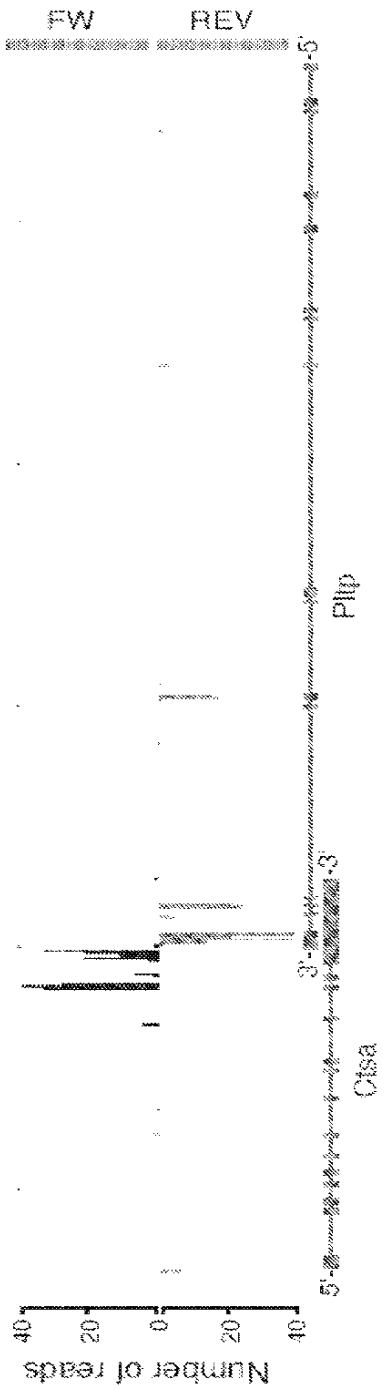


Figure 15

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01027

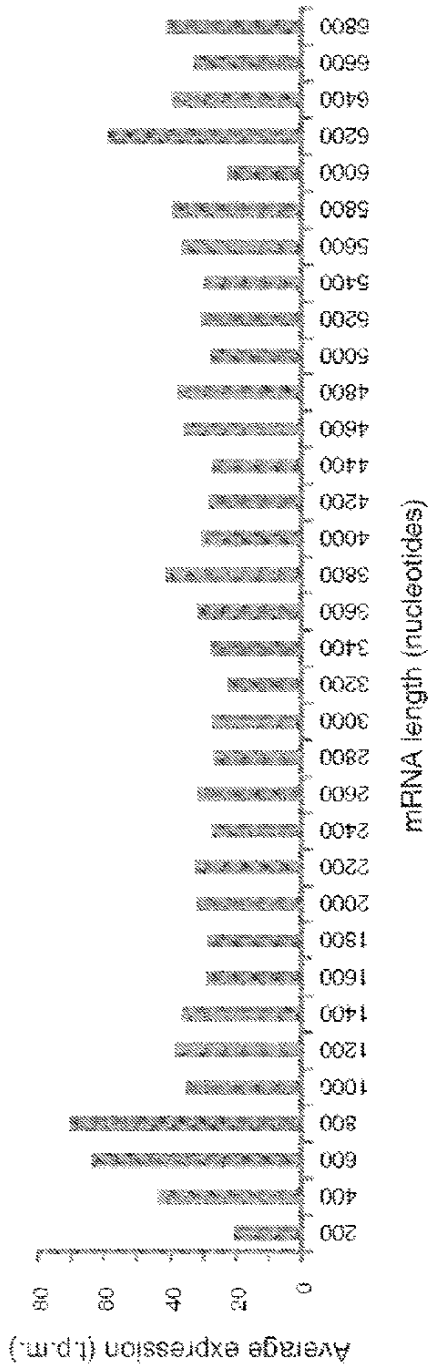


Figure 16

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01028

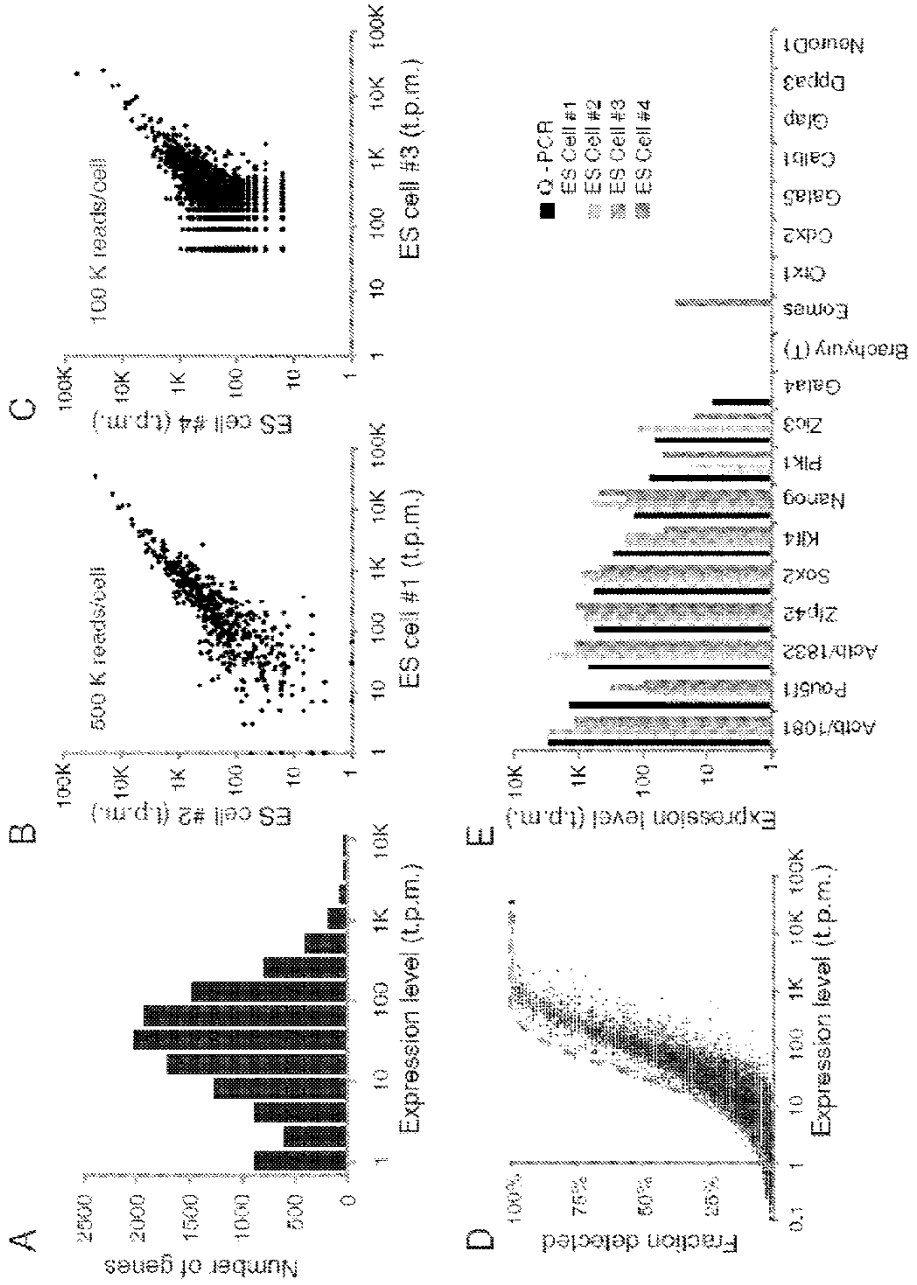


Figure 17

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01029

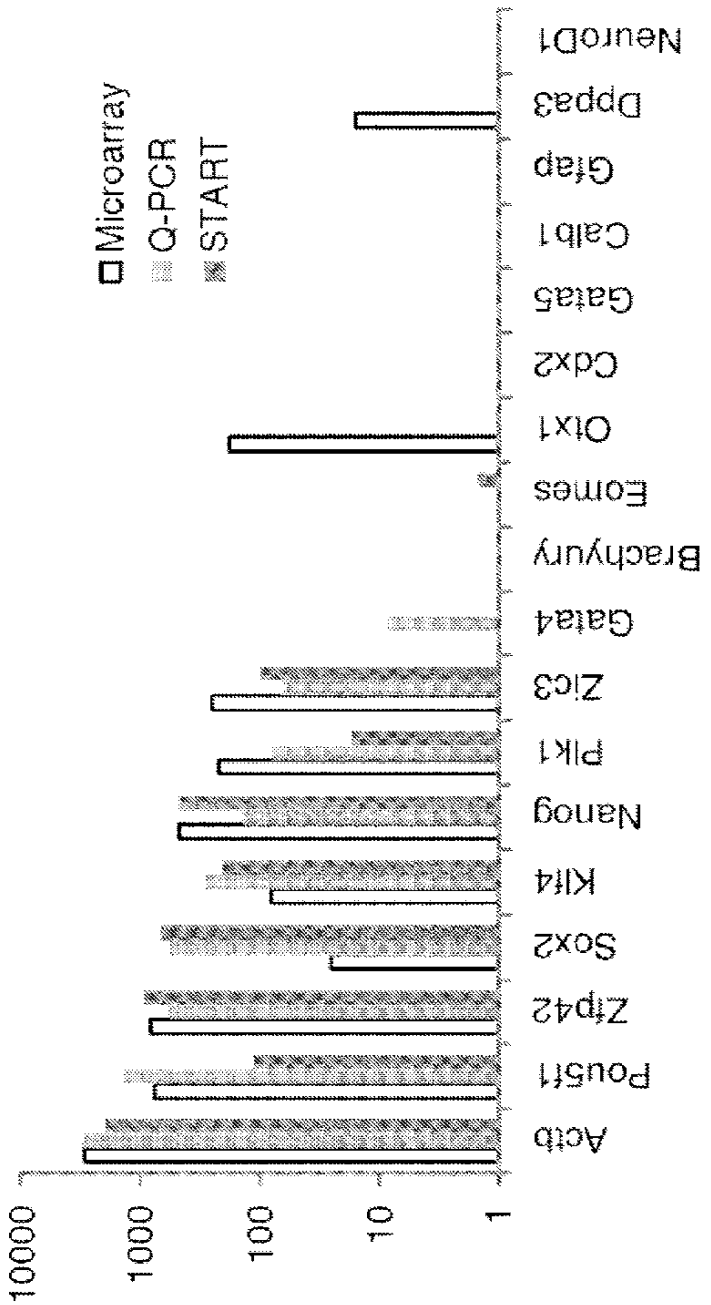


Figure 18

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01030

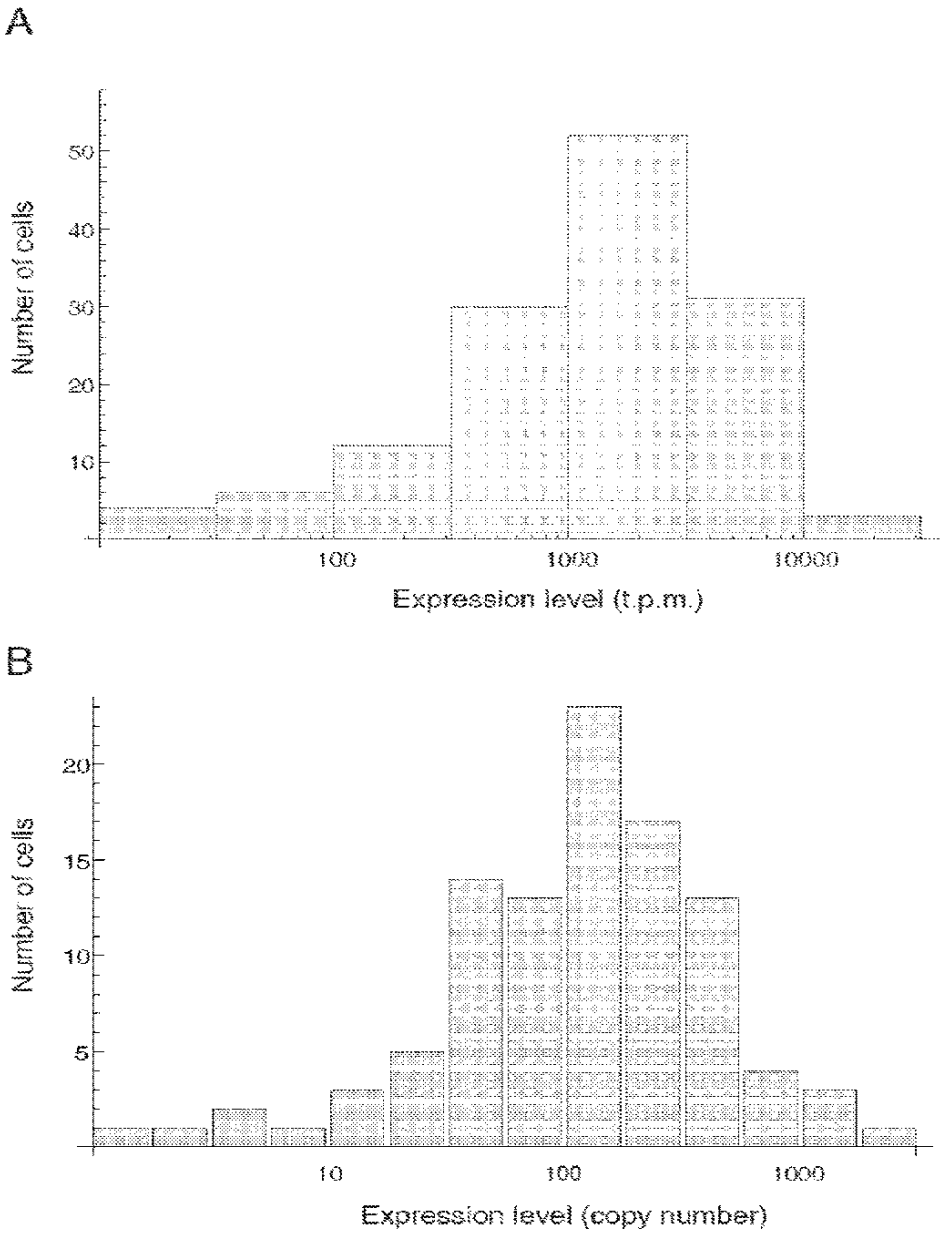


Figure 19

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01031

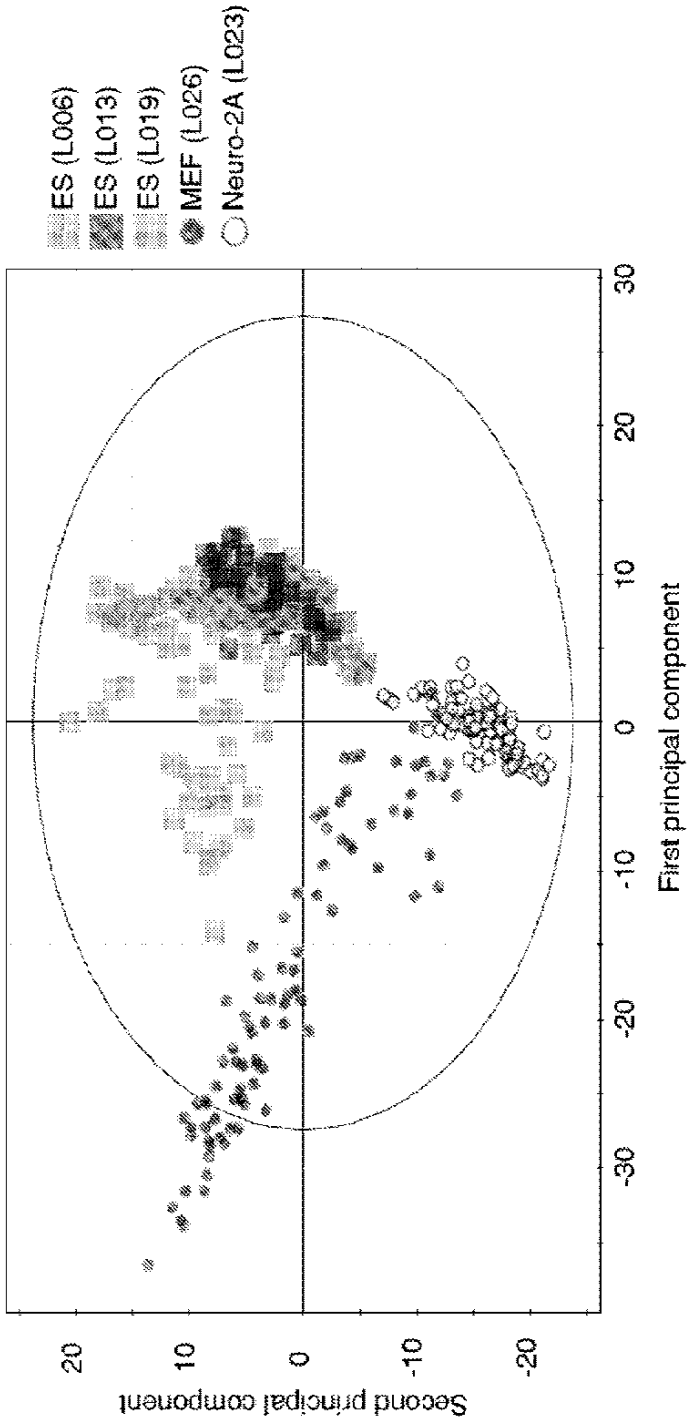


Figure 20

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 01032

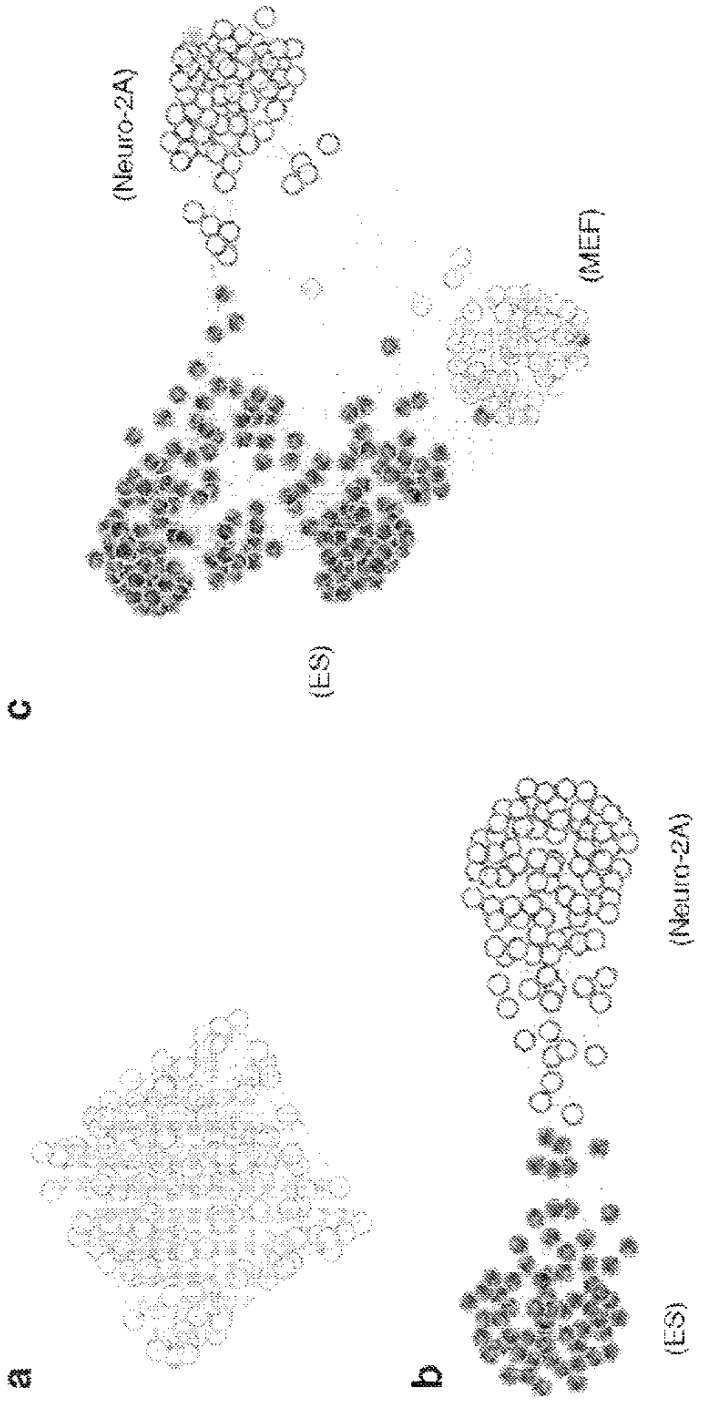


Figure 21

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 01033

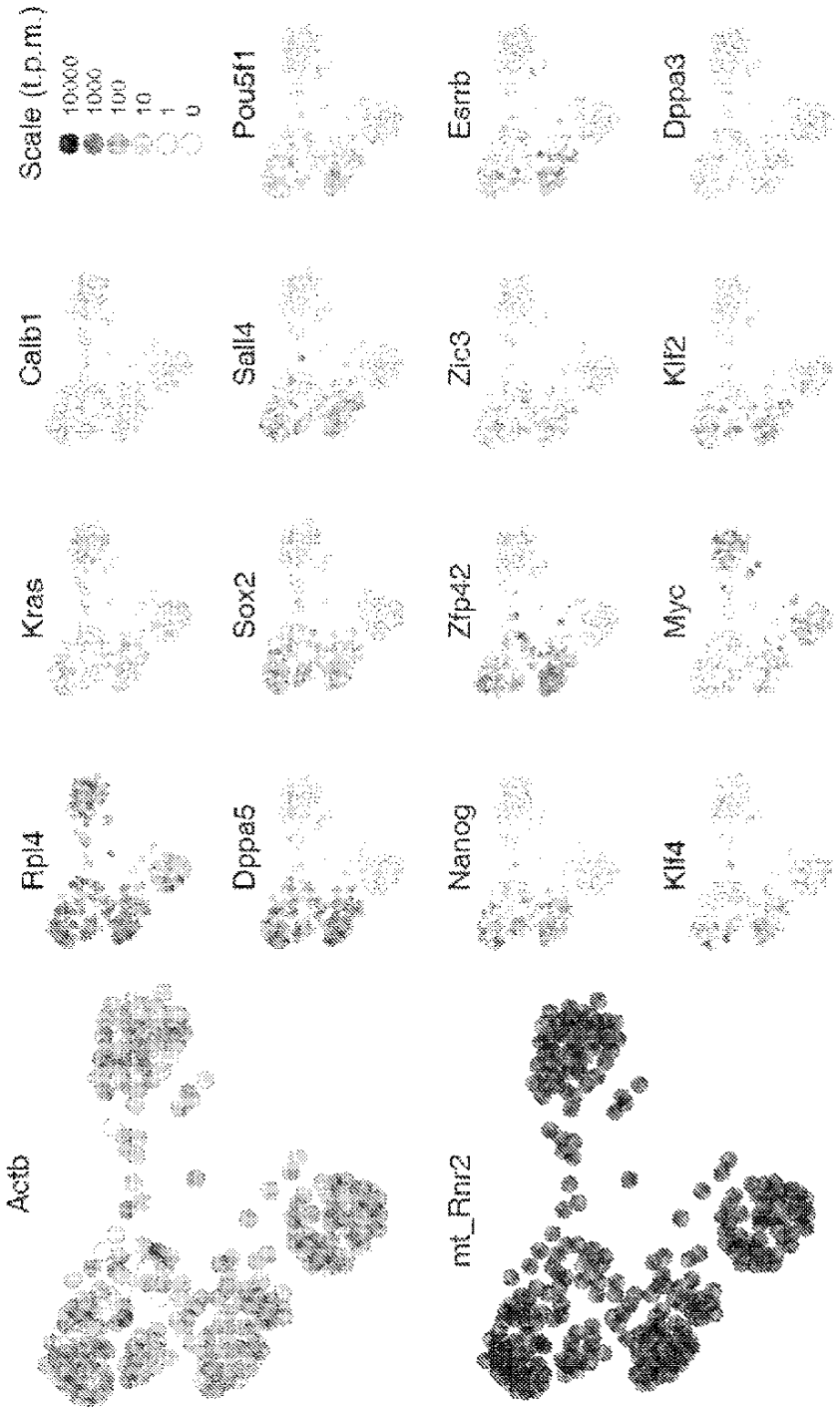


Figure 22

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01034

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Conf. No.: **9697**
Sidney BRENNER	Art Unit: **1634**
Application No.: **14/792,094**	Examiner: **Narayan Kameshwar Bhat**
Filed: **July 6, 2015**	Docket No.: **104371-5016-US05**
For: **Compositions and Methods for Intramolecular Nucleic Acid Rearrangement**	*Certificate of Transmission (37 C.F.R. § 1.8)* *I hereby certify that this correspondence is being transmitted to the U.S. Patent and Trademark Office via the Office electronic filing system (EFS-Web) to the USPTO on August 30, 2017.* / Sheila R. Gloss / *Sheila R. Gloss*

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

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Commissioner:

This Information Disclosure Statement is hereby submitted in accordance with 37 C.F.R. 1.98 and pursuant to Applicant's continuing duty under 37 C.F.R. 1.56 to bring any information which may be material to patentability of this application to the Examiner's attention.

Applicant makes no representation that a search has been conducted by the Applicant, or that there is not possibly more relevant art. 37 C.F.R. § 1.97(g). Applicant also makes no representation that the information submitted herewith is in fact material to patentability. The filing of this Information Disclosure Statement shall not be construed as an admission against interest in any manner. 37 C.F.R. § 1.97(h).

The Examiner's attention is directed to the reference(s) cited on the accompanying substitute for forms 1449A/PTO and PTO/SB/08. Except for U.S. Patents and U.S. Patent Application Publications, *see* 37 C.F.R. § 1.98(a)(2)(i), copies of the cited references are enclosed, unless otherwise noted below.

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Page 01035

This Information Disclosure Statement is being filed within three months of the filing date of a national application other than a continued prosecution application, within three months of the date of entry of a national stage, before the mailing of a first Office action on the merits, or before the mailing of a first Office action after the filing of a request for continued examination. 37 C.F.R. § 1.97(b). No fee is required.

The Commissioner is hereby authorized to charge any underpayment of the following fees associated with this communication, including any necessary fees for the submission of an information disclosure statement under 37 CFR § 1.17(p) or for the extension of time, or credit any overpayment to Deposit Account No. 50-0310.

Respectfully submitted,

Date: August 30, 2017

By: /Gargi Talukder/
Gargi Talukder, Reg. No. 61,368
Filed Under 37 C.F.R. § 1.34

MORGAN LEWIS & BOCKIUS LLP
One Market, Spear Street Tower
San Francisco, California 94105
Tel: 415.442.1000
Fax: 415.442.1001

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01036

Substitute for form 1449A/PTO (Substitute PTO/SB/8A)				Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	**14/792,094**
				Filing Date	**July 6, 2015**
				First Named Inventor	**Sidney BRENNER**
(use as many sheets as necessary)				Art Unit	**1634**
				Examiner Name	**Narayan Kameshwar Bhat**
Sheet	1	of	1	Attorney Docket Number	**104371-5016-US05**

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	U.S. Patent Document Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	A1	US 2006/0177833A1	08-10-2006	Brenner	
	A2	US 7,329,493 B2	02-12-2008	Chou et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[2] Number[3] Kind Code[4] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[5]
	A3	WO 2007147079 A2	12-21-2007	Living Microsystems, Inc. et al.		
	A4	WO 2010117620 A3	10-14-2010	Illumina, Inc.		

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[6]
	A5	"Using Multiplex Identifier (MID) Adaptors for the GS FLX Titanium Chemistry Basic MID Set" Genome Sequencer FLX System, Technnical Bulletin 004-2009, (Apr 1 2009) pp 1-7. URL:http://454.com/downloads/my454/documentation/technical-bulletins/TCB-09004 Using MultiplexIdentifierAdaptorsForTheGSFLXTitaniumSeriesChemistry-BasicMIDSet.pdf	
	A6	"Using Multiplex Identifier (MID) Adaptors for the GS FLX Titanium Chemistry Extended MID Set" Genome Sequencer FLX System, Technnical Bulletin 005-2009, (Apr 1 2009) pp 1-7. URL:http://454.com/downloads/my454/documentation/technical-bulletins/TCB-09005 UsingMultiplexIdentifierAdaptorsForTheGSFLXTitaniumChemistry-ExtendedMIDSet.pdf	
	A7	AHERN, "Biochemical, Reagents Kits Offer Scientists Good Return on Investment" The Scientist (1995) 9(15):1-7	
	A8	REPP et al. "Genotyping by Multiplex Polymerase Chain Reaction for Detection of Endemic Hepatitis B Virus Transmission" J Clinical Microbiology (1993) 31:1095-1102	
	A9	WISEMAN, R.W. et al. "Major histocompatibility complex genotyping with massively parallel pyrosequencing" Nature Medicine (Oct 11 2009) 15(11):1322-1326	
	A10	Xiao, et al, "Determination of haplotypes from single DNA molecules: a method for single-molecule barcoding", Hum Mutat., 28:913-21, 2007.	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.[1] Applicant's unique citation designation number (optional).[2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04.[3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3).[4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document.[5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible.[6] Applicant is to place a check mark here if English language Translation is attached.

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

DB2/ 31885247.1

Electronic Patent Application Fee Transmittal

Application Number:	14792094
Filing Date:	06-Jul-2015
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Filer:	Gargi Talukder/Sheila Gloss
Attorney Docket Number:	104371-5016-US05

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($)
Miscellaneous:				
RCE- 2ND AND SUBSEQUENT REQUEST	2820	1	850	850
		Total in USD ($)		**850**

Electronic Acknowledgement Receipt	
EFS ID:	30232442
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	43850
Filer:	Gargi Talukder/Sheila Gloss
Filer Authorized By:	Gargi Talukder
Attorney Docket Number:	104371-5016-US05
Receipt Date:	30-AUG-2017
Filing Date:	06-JUL-2015
Time Stamp:	19:53:45
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$850
RAM confirmation Number	083117INTEFSW00006271500310
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Continued Examination (RCE)	104371-5016-US05_RCE.pdf	1349886 1ace27577aa3c6e491951674a4acdd80017d2982	no	3
Warnings:					
Information:					
2		104371-5016-US05_Response.pdf	180562 470384200d4172a31a65caa8d585c0064fc55568d5	yes	6
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Response After Final Action		1	1	
	Claims		2	3	
	Applicant Arguments/Remarks Made in an Amendment		4	6	
Warnings:					
Information:					
3	Foreign Reference	WO2007147079-PAMPH-618.pdf	6657853 31e9a83321056aa6ee9e5679aa65560f62779c2	no	99
Warnings:					
Information:					
4	Foreign Reference	WO2010117620-PAMPH-142.pdf	7973504 5aac1ce727109a9f320ddaee6d025612037c70c7	no	71
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Information:					
5	Non Patent Literature	BasicMIDSet.pdf	614106 30f6a9911ea35fcdc55968f0f6646644eac099c13	no	11

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Information:					
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Information:					
7	Non Patent Literature	AHERN.pdf	538716 c2020218a32c32aacc9d1af1df399d10650ce65	no	7
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Information:					
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Information:					
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Warnings:					

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Total Files Size (in bytes):	21060885

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (09-11)
Approved for use through 1/31/2014 OMB 0651-0032
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/792,094	Filing Date 07/06/2015	☐ 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	140
☐ SEARCH FEE (37 CFR 1.16(k), (l), 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	140

APPLICATION AS AMENDED – PART II

AMENDMENT	08/30/2017	(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))	* 9	Minus	** 20	= 0	X $40 =	0
	Independent (37 CFR 1.16(h))	* 1	Minus	***3	= 0	X $210 =	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".
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				Filing Date	**July 6, 2015**
				First Named Inventor	**Sidney BRENNER**
(use as many sheets as necessary)				Art Unit	**1634**
				Examiner Name	**Narayan Kameshwar Bhat**
Sheet	1	of	1	Attorney Docket Number	**104371-5016-US05**

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	A1	US 2006/0177833A1	08-10-2006	Brenner	
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DJ Lockhart, EA Winzeler - Nature, 2000 - search.proquest.com
... Now that **nucleic** acid arrays have been constructed for many different organisms14,2629 and ... strain corresponds to a specific location on an array that contains **oligonucleotide** probes that ... first is a PCR-based approach that has been used to make **single-cell cDNA** libraries7678 ...
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... 4. The bio-**barcode** assay: homogeneous meets heterogeneous. The polymerase chain reaction (PCR), when set up properly, is powerful enough to amplify a target **nucleic** acid sequence from even a **single cell**, such that large quantities are available for downstream ...
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Single-cell gene expression profiling
JM Levsky, SM Shenoy, RC Pezo, RH Singer - Science, 2002 - science.sciencemag.org
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A bio-bar-code assay based upon dithiothreitol-induced **oligonucleotide** release
CS Thaxton, HD Hill, DG Georganopoulou... - Analytical ..., 2005 - ACS Publications
... **nucleic** acid targets has been shown to be extraordinarily sensitive, exhibiting low attomolar sensitivity for protein targets and high zeptomolar sensitivity for **nucleic** acid targets. In the case of DNA detection, the original assay relies on three distinct **oligonucleotide** strands on a ...
Cited by 175 Related articles All 6 versions Cite Save

Immuno-PCR: high sensitivity detection of proteins by **nucleic** acid amplification
CM Niemeyer, M Adler, R Wacker - TRENDS in Biotechnology, 2005 - Elsevier
... In immuno-rolling circle amplification (RCA) technology, an **oligonucleotide** primer, covalently attached to the ... been intensively investigated for their material properties and applications in **nucleic** acid analysis ... from the gold nanoparticle, and the released DNA **barcode** is used to ...
Cited by 295 Related articles All 8 versions Cite Save

Molecular encoding of **nucleic** acid templates for PCR and other forms of sequence analysis
M McCloskey, C Laird, R Hansen, R Stoger... - US Patent App. 11/ ..., 2005 - Google Patents
... under suitable conditions to anneal the bar-coded **oligonucleotide** to the target **nucleic** acid molecule, wherein the bar-coded **oligonucleotide** comprises a first sequence complementary to the target **nucleic** acid molecule, a second sequence providing a random **barcode**, and a ...
Cited by 50 Related articles All 2 versions Cite Save

Nanoparticles and their biological and environmental applications
WT Liu - Journal of Bioscience and bioengineering, 2006 - Elsevier
... Usually, they are derivatized with different functional groups such as **nucleic** acid-targeted ... specific DNA duplex were released into solution and the amount of **oligonucleotide** released was ... can be further developed to achieve rapid bacterial detection at the **single-cell** level (35). ...
Cited by 306 Related articles All 16 versions Cite Save

Encoding PCR products with batch-stamps and barcodes
ML McCloskey, R Stöger, RS Hansen, CD Laird - Biochemical genetics, 2007 - Springer
... by which molecular codes are added to single-stranded DNA or **cDNA** templates, thus ... be used when amplifying irreplaceable DNAs and cDNAs for forensic, clinical, **single cell**, and ancient ... **Nucleic** Acids Res 32(17):e135PubMedCrossRefGoogle Scholar. Stöger R, Kajimura TM ...
Cited by 22 Related articles All 13 versions Cite Save

Cancer nanotechnology: opportunities and challenges
M Ferrari - Nature reviews. Cancer, 2005 - search.proquest.com
... as a result of affinity binding and as a result of **nucleic**-acid hybridization ... precise map of the distribution of many molecular markers in a **single cell**, cell population or ... developed by Chad Mirkin and colleagues10.Dubbed bio-**barcode**, it involves **oligonucleotide**-modified gold ...
Cited by 3580 Related articles All 11 versions Cite Save

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 01064

Miniaturised **nucleic** acid analysis
PA Auroux, Y Koc, A Manz, PJR Day - Lab on a Chip, 2004 - pubs.rsc.org
... comparing poly(A) tails and random hexamers for **cDNA** synthesis were ... applied dielectrophoresis and electrical impedance spectroscopy for **single cell** manipulation within microfluidic ... DNAzyme, a catalytically active **oligonucleotide** capable of cleaving **nucleic** acid substrates ...
Cited by 251 Related articles All 3 versions Cite Save

Efficient microRNA capture and bar-coding via enzymatic **oligonucleotide** adenylation
F Vigneault, AM Sismour, GM Church - Nature methods, 2008 - nature.com
... RNA or DNA-RNA chimera), and observed that the RNA and chimera adaptor successfully ligated to the 5' end of the synthetic miRNA whereas the DNA **oligonucleotide** did not ... **Nucleic** Acids Res. ... Systematic and **single cell** analysis of Xenopus Piwi-interacting RNAs and Xiwi ...
Cited by 51 Related articles All 10 versions Cite Save

The current status of **cDNA** cloning
M Harbers - Genomics, 2008 - Elsevier
... **cDNA** synthesis from whole RNA is primed by an **oligonucleotide** containing a T7 RNA ... procedure have been published to enable full-length **cDNA** cloning after **cDNA** tailing by ... An alternative approach to **single-cell cDNA** library preparation makes use of oligo(dT) primers linked ...
Cited by 37 Related articles All 7 versions Cite Save

Technology for microarray analysis of gene expression
A Watson, A Mazumder, M Stewart... - Current opinion in ..., 1998 - Elsevier
... K, Hubert R, Navidi W, Arnheim N: Whole genome amplification from a **single cell**: implications for ... Wetmur JG: DNA probes: applications of the principles of **nucleic** acid hybridization. ... expression in the murine thymus assayed by quantitative hybridization of arrayed **cDNA** clones ...
Cited by 186 Related articles All 6 versions Cite Save

Mammalian RNAi: a practical guide
P Sandy, A Ventura, T Jacks - Biotechniques, 2005 - researchgate.net
... red label for control cells) and the products are hybridized to the **barcode oligonucleotide** array (4 ... The **cDNA** of a target gene flanked by recombination (att) sites is cloned downstream of ... cassettes can be directly injected into the perivi- telline space of **single-cell** mouse embryos ...
Cited by 188 Related articles All 13 versions Cite Save More

From **single cell** gene-based diagnostics to diagnostic genomics: current applications and future perspectives
R Zhao - Clinical Laboratory Science, 2005 - search.proquest.com
... of genomic and proteomic approaches especially in pure cell types or even in the **single-cell** level, which ... **Nucleic** acid sequence based amplification (NASBA). ... reverse transcription from an RNA template by AMV reverse transcriptase and RNA amplification from **cDNA** by gene ...
Cited by 11 Related articles All 6 versions Cite Save

Methods, flow cells and systems for **single cell** analysis
R Joseph, JJ DiMeo, M Jarosz... - US Patent App. 12/..., 2009 - Google Patents
... Methods for preparing a **nucleic** acid sample from a **single cell** and capturing **nucleic** acids ... function as both surface capture and sequencing primers, especially when an **oligonucleotide** containing a ... Optionally, the sample **nucleic** acid fragments can be attached to a surface via ...
Cited by 15 Related articles All 4 versions Cite Save

Homogeneous methods for **nucleic** acid amplification and detection
RG Higuchi - US Patent 5,994,056, 1999 - Google Patents
... a sample is provided which contains, or is suspected of containing, a particular **oligonucleotide** sequence of ... For example, in a PCR method where the target **nucleic** acid is single-stranded DNA, such as mRNA, the target may be first reverse-transcribed into **cDNA**, prior to ...
Cited by 266 Related articles All 2 versions Cite Save

Multiplexed and microparticle-based analyses: Quantitative tools for the large-scale analysis of biological systems
JP Nolan, F Mandy - Cytometry Part A, 2006 - Wiley Online Library
... hybridization to any other **oligonucleotide** in the set (54, 55), Microspheres bearing such **oligonucleotide** tags offer ... The examples described earlier for immunoassays and **nucleic** acid analyses represent the best-developed uses of ... Quantitative **single cell** analysis and sorting. ...
Cited by 122 Related articles All 8 versions Cite Save

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01065

Microfluidic device for analyzing **nucleic** acids and/or proteins, methods of preparation and uses thereof

F Chatelain, FW Frueh - US Patent App. 10/139,100, 2002 - Google Patents

... a 3-ethoxy-aminopropyl-silanol group, from which will be extended the **oligonucleotide** chain terminating **...** [0093]. The present invention thus relates to a method for analyzing **nucleic** acids and **...** in very small amounts, such as when working with extracts obtained from a **single cell**. **...**

Cited by 28 Related articles All 2 versions Cite Save

Single-cell fluorescence imaging using metal plasmon-coupled probe 2: single-molecule counting on lifetime image

J Zhang, Y Fu, D Liang, K Nowaczyk, RY Zhao... - Nano ..., 2008 - ACS Publications

... Single-Cell Fluorescence Imaging Using Metal Plasmon-Coupled Probe 2: Single-Molecule Counting on Lifetime Image. **...**

Cited by 56 Related articles All 11 versions Cite Save

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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	14828	(barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3) near5 (distinct or different).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/13 08:57
L2	6654	(cdna or nucleic or dna or polynucleotide)same (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/13 08:58
L3	0	2 and (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3)same (different or distinct) same each.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/13 08:59
L4	1574	2 and (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3)same (different or distinct).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/13 09:00
L5	52	4 and "single cell".clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/13 09:00
L6	96	2 and "single cell".clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/13 09:04
L7	13	6 and pool$3 near5 (nucleic or dna or polynucleotide or cdna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/13 09:05
S1	1445	illumina.as. or linnarsson.in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/12 18:25
S2	6	S1 and "single cell" same (mrna or nucleic or dna or polynucleotide or	US-PGPUB; USPAT; USOCR;	OR	ON	2017/09/12 18:27

		ribonucleic).clm.	FPRS; EPO; JPO; DERWENT; IBM_TDB			
S3	30651	"10X GENOMICS".as. or brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "population genetics technologies".as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/12 21:39
S4	3	S3 and "single cell" same (mrna or nucleic or dna or polynucleotide or ribonucleic).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/12 21:40
S5	343	"single cell" same (mrna or nucleic or dna or polynucleotide or ribonucleic).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/12 21:41
S6	6	S5 and (mid or "multiplex identifier").clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/12 21:42
S7	4833	"single cell" near5 (mrna or nucleic or dna or polynucleotide or ribonucleic)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/12 21:44
S8	219	S7 and (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3) near5 "single cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/12 21:46
S9	166	S8 and (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3) near5 (mrna or nucleic or dna or polynucleotide or ribonucleic)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/12 21:48
S10	118	S9 and (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3) near5 (distinct or different)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/12 21:48

9/13/2017 9:06:12 AM
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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	4	("10X GENOMICS".as. or brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "population genetics technologies".as.) and ("single cell" or "one cell" or "individual cell") same (nucleic or dna or polynucleotide or cdna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/14 08:57
L2	89	("10X GENOMICS".as. or brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "population genetics technologies".as.) and (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3) same (nucleic or dna or polynucleotide or cdna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/14 09:00
L3	4	2 and ("single cell" or "one cell" or "individual cell") same (nucleic or dna or polynucleotide or cdna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/14 09:01
L4	1	2 and ("single cell" or "one cell" or "individual cell") same (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3) same (identif$6 or specific).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/14 09:04
L5	1	("10X GENOMICS".as. or brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "population genetics technologies".as.) and ("single cell" or "one cell" or "individual cell") same ("single cell" or "one cell" or "individual cell") same (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3) same (identif$6 or specific).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/14 09:05
L6	176	("single cell" or "one cell" or "individual cell") same ("single cell" or "one cell" or "individual cell") same (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3) same (identif$6 or specific).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/14 09:06
L7	36	6 and (nucleic or dna or cdna) same (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3) same (identif$6 or specific).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/14 09:09

L8	671	"cell specific" near5 (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/14 09:20
L9	25	8 and (dna or nucleic or cdna or polynucleotide) near5 specific near5 (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/14 09:22

9/14/2017 9:27:58 AM
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/792,094	07/06/2015	Sydney Brenner	104371-5016-US05	9697

43850 7590 09/20/2017
MORGAN, LEWIS & BOCKIUS LLP (SF)
One Market, Spear Street Tower, Suite 2800
San Francisco, CA 94105

EXAMINER
BHAT, NARAYAN KAMESHWAR

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
09/20/2017	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):

sfipdocketing@morganlewis.com
donald.mixon@morganlewis.com

PTOL-90A (Rev. 04/07)

Office Action Summary	Application No. 14/792,094	Applicant(s) Brenner et al.	
	Examiner NARAYAN BHAT	Art Unit 1634	AIA Status No

-- 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 8/30/2017 .

☐ 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) 56-64 is/are pending in the application.

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

6)☐ Claim(s) _____ is/are allowed.

7)☑ Claim(s) 56-64 is/are rejected.

8)☑ Claim(s) 63-64 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 06 July 2015 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: _____ .

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Continued Examination under 37 CFR 1.114

1. 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 8/30/2017 has been entered.

2. The present application is being examined under the pre-AIA first to invent provisions. The instant application is a continuation of 14/172,694 now U.S. Patent 9,102,980, which is a continuation of 14/021,790, now U.S. Patent 8,679,756, which is a continuation of 13/859,450 now U.S. Patent 8,563,274, which is a continuation of 13/622,872 now abandoned, which is a continuation of 13/387,343, now U.S. Patent 8,298,767, which is a national stage entry of PCT/IB2010/002243, filed on 08/13/2010, claiming priorities from provisional applications 61288792 filed on 12/21/2009, 61235595 filed on 08/20/2009.

It is noted that none of the provisional applications provide support for the limitation of "*second multiplex identifier sequences are all different from each other*". *Therefore the priority for the instant application is 08/13/2010*.

3. This office action is in response to the papers filed on 8/30/2017.

Track1 request

4. Track1 request filed by the applicant on December 22, 2016 has been granted by the office on January 10, 2017. Therefore instant application has been examined on

a priority basis. To maintain its status applicant is required to follow the requirements listed in the Track 1 grant document.

Interview summary

5. In view of instant application being part of family of patents, for compact prosecution, the examiner contacted the applicant's representative Ms. Talukder telephonically on 9/8/2017 to inform that further amendments are needed to overcome the potential rejections under 35 USC 112 first and second paragraphs and to fix the minor informalities.

The examiner suggested to further clarifications in claim 56 are needed to specify that the plurality of nucleic acid segments are made from each of the single isolated cell, wherein the plurality of nucleic acid segments from each single cell have cell specific multiplex identifier and different sizes of the nucleic acids from each cell have different multiplex identifier consistent with results schematically presented in figures 11 and 12 (top panel). The examiner also informed that claim 56 as recited is not clear how plurality of nucleic acid samples from plurality of single isolated cell being tagged with first and second multiplex identifiers capable identifying origin of nucleic acid sample each of the single isolated cell.

The amendments to include the step of isolating nucleic acids from each of the isolated single cell separately and appending nucleic acid sample with cell specific identifier were discussed. But no specific amendments to clarify how second MID (multiplex identifier) are different from each other were discussed in view of 35 USC 112 rejections are not being on the record. In view of time constraint to move the instant

application on an expedited basis on the part of the examiner and to get specific claim language approval from the applicant, the examiner informed that the office action will be issued to decide further course of action. No specific claim languages were agreed to during the interview.

Claim status

6. In the claim listing of 8/30/2017 claims 56-64 are pending in this application and are under prosecution. Claims 56-64 are new. Claims 1-55 are canceled. The claim amendments have been reviewed and entered.

Withdrawn Rejections and Response to the Remarks

6. The previous rejection of claims 40-55 under pre-AIA 35 USC 103(a) as being unpatentable over Gillevet in view of Wangh has been withdrawn in view of cancelation of said claims. The arguments regarding the teachings of Gillevet and Wangh have been fully considered (Remarks, pgs. 4 and 5). They are moot in view of withdrawn rejection necessitated by the cancelation of said claims.

7. All pending ODP rejections on the record have been withdrawn in view of cancellation of instant claims 11-44.

However, upon further consideration, new grounds of rejections are set forth in this office action as discussed below.

Claim Objections

8. Claims 63 and 64 are objected to because of the following informalities.

Claim 63 is objected over the recitation of "the segments of (a)" in line 1 and appears to be minor typographical error because step 'a' of claim 56 is drawn to nucleic acid samples not to segments.

Claim 64 is dependent from canceled claim 40 and appears to be a minor typographical error. For compact prosecution, claim 64 has been presumed to be dependent from claim 56. In response to this office action, applicant is suggested to identify the proper dependency of claim 64.

Appropriate corrections are required.

Claim Rejections - 35 USC § 112 first paragraph

Scope of Enablement Rejection

9. 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.

10. 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.

11. Claims 56-64 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification, while being enabled for nucleic acid sample

made from each of the single isolated cell, wherein the nucleic acid sample from each single isolated cell tagged with unique/distinct first multiplex identifier (first MID) sequence to track the origin of nucleic acid samples, wherein the different sized nucleic acid segments from plurality of nucleic acid samples from plurality of isolated single cells are tagged with different second MID sequences, to distinguish different sized nucleic acid segments (as shown in figures 11 and 13). However, the specification is not enabled for (a) providing a plurality of nucleic acid samples, each made from single isolated cells; (b) tagging a plurality of segments of the nucleic acid molecules in the samples of (a) with first and second multiplex identifier (MID) sequences, wherein the second MID sequences are all different from each other.

The instant specification discloses "In certain embodiments, the nucleic acid sample being analyzed is derived from a single source (e.g., a single organism, virus, tissue, cell, subject, etc.), whereas in other embodiments, the nucleic acid sample is a pool of nucleic acids extracted from a plurality of sources (e.g., a pool of nucleic acids from a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more" (pg. 19, paragraph 2) and "In certain embodiments, nucleic acid fragments that are to be pooled with nucleic acid fragments derived from a plurality of sources (e.g., a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. In such embodiments, the nucleic acids derived from each source includes a multiplex identifier (MID) such that the source from which the each tagged nucleic acid fragment was derived can be determined (pg. 19 paragraph 3) and "In certain embodiments, each individual polynucleotide (e.g., double-stranded or single-stranded, as appropriate to the methodological details employed) in a sample to be analyzed is

tagged with a unique MID so that the fate of each polynucleotide can be tracked in subsequent processes (where, as noted above, unique MID is meant to indicate that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID). For example (and as described below), having each nucleic acid tagged with a unique MID allows analysis of the sequence of each individual nucleic acid using the reflex sequence methods described herein. This allows the linkage of sequence information for large nucleic acid fragments that cannot be sequenced in a single sequencing run" (pg. 20, paragraph 1, emphasis under lined by the examiner). In other words to enable the method of claim 56, reflex sequence method is essential for tagging different sized nucleic acid segments with different second MID sequences.

Therefore the instant specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, the invention requiring the steps of (a) providing a plurality of nucleic acid samples, each made from single isolated cells; (b) tagging a plurality of segments of the nucleic acid molecules in the samples of (a) with first and second multiplex identifier (MID) sequences, wherein the second MID sequences are all different from each other (recited in generality). Therefore instant specification is not enabled for carry out the method of claim 56 as discussed in detail below.

Per eMPEP 2164.01(a) there are many factors to be considered when determining whether there is sufficient evidence to support a determination that a disclosure does not satisfy the enablement requirement and whether any necessary experimentation is "undue." These factors include, but are not limited to:

(A) The breadth of the claims;

(B) The nature of the invention;

(C) The state of the prior art;

(D) The level of one of ordinary skill;

(E) The level of predictability in the art;

(F) The amount of direction provided by the inventor;

(G) The existence of working examples; and

(H) The quantity of experimentation needed to make or use the invention based on the content of the disclosure.

Breadth of Claims: In the instant case, claim 56 as recited encompass large number of single isolated cells, which will have very large number of nucleic acid samples and appending first MID and second MID sequences to large number of nucleic acid segments and sequencing appended segments which generate large number of sequences reads and not able to even analyze which first MID sequence of nucleic acid sample is from single isolated cell number 1 versus cell number "n" because of presence of large number of nucleic acid samples appended with first MID sequence. Also, instant claim 56 as recited encompasses a large genus of single isolated cells, plurality of nucleic acid samples and large number of nucleic acid segments tagged with first MID sequence and different second MID sequences.

Nature of the invention and state of the prior art: The instantly claimed invention is directed to analyze nucleic acids from single isolated cell and matching the nucleic acid sequences originating from each of the nucleic acid using reflex method to introduce a plurality of multiplex identifiers in plurality of nucleic acid segments.

Tagging nucleic acid samples with sample specific MID sequence or isolating single cells, tagging nucleic acid sample from each isolated single cell with specific first MID sequence to track the origin of nucleic acid sample from each of isolated single cell and pooling the nucleic acids and identifying the nucleic acid from each sample based on the sample or cell specific first MID sequence were known in the art at the time the claimed invention was made (Gillevet et al, arts of the record; Fig. 1 and Linnarsson, cited below, claims 1-5).

However, the step of tagging a plurality of segments of the nucleic acid molecules in the samples from plurality of single isolated cells with first and second multiplex identifier (MID) sequences, wherein: (i) the first MID sequences associated with a segment of a nucleic acid molecule of (a) uniquely identifies the single isolated cell from which the nucleic acid molecule is provided; and (ii) the second MID sequences are all different from each other was not established in the art prior to the claimed invention. Generation of large number of MID sequences were also known at the time of the claimed invention (454 multiplex identifier sequence, Roche bulletin, cited in the IDS of 8/30/17).

The level of one of ordinary skill: The level of skills required is very high as the claimed method requires isolating single cell, nucleic acids from the cell, tagging, and sequencing of the tagged nucleic acids and analysis of the sequence data to trace back the origin of nucleic acid from each cell.

The amount of direction provided by the inventor and the existence of working examples: The instant specification set forth steps to append tags to nucleic acid sample from each sample or a cell and isolating nucleic acids and appending the

nucleic acids with different multiplex identifier sequences using Reflex method (Fig. 1) and generating nucleic acid segments using primers/adaptors and specifically appending different multiplex identifiers to different sizes of fragments (Figs. 11 and 13 and their descriptions in the text).

The instant specification does not provide guidance regarding step 'b' of instant claim 56, especially tagging plurality of segments of the plurality of nucleic acid samples with first and different second MID sequences and identifying the origin of nucleic acid segments with specific isolated single cell.

The level of predictability in the art and the quantity of experimentation needed:

One having ordinary skill in the art would recognize that it is not possible to identify the source of nucleic acid from each sample when all of the plurality of the samples have the same first MID sequences. Therefore quantities of experimentations to be done are very large especially when the instant specification does not provide any guidance for addressing issues as discussed especially step 'b' of claim 56 in the previous sections.

Conclusion:

Courts have ruled that to be enabling, the specification of a patent must teach those skilled in the art how to make and use the full scope of the claimed invention without undue experimentation (In re Wright990 F.2d 1557, 1561; In re Fisher, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970)). It was also determined that the scope of the claims must bear a reasonable correlation to the scope of enablement provided by the specification to persons of ordinary skill in the art. The amount of guidance needed to enable the invention is related to the amount of knowledge in the art as well as the

predictability in the art. Furthermore, the Court in Genentech Inc. v Novo Nordisk 42 USPQ2d 1001 held that it is the specification, not the knowledge of one skilled in the art that must supply the novel aspects of the invention in order to constitute adequate enablement.

Considering all the factors discussed above and lack of working example for the steps of claim 56 it is clear that the specification does not disclose a representative number of working examples. Thus, one skilled in the art would be lead to conclude that the applicant was not in possession of the claimed step 'b' encompassing large number of genus.

Therefore the claimed method is enabled for nucleic acid sample made from each of the single isolated cell, wherein the nucleic acid sample from each single isolated cell tagged with unique/distinct first multiplex identifier (first MID) sequence to track the origin of nucleic acid samples, wherein the different sized nucleic acid segments from plurality of nucleic acid samples from plurality of isolated single cells are tagged with different second MID sequences consistent with the reflex method of tagging MID sequences.

12. Claims 57-64 are rejected under pre-AIA 35 USC 112 first paragraph because they include all the limitations of claim 56.

For compact prosecution, applicant is suggested to identify support for the amendments in the instant specification.

Claim Rejections - 35 USC § 112-second paragraph

13. 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.

14. 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.

15. Claims 56-64 are 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.

16. Claim 56 is rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being incomplete for omitting essential steps, such omission amounting to a gap between the steps (See MPEP § 2172.01). The omitted steps are: (i) the details of the steps of tagging first and second MID sequences to the plurality of segments of the nucleic acid molecules from the plurality of nucleic acid samples from plurality of single isolated cells, (ii) not specifying the step of tagging nucleic acid molecules from, for example, single isolated cell 1 with first MID sequences type 1 separately from the step of tagging nucleic acid molecules from single isolated cell 2 with first MID sequences type 2 and (iii) not specifying the step of how plurality of tagged segments of nucleic acid molecules with first MID sequences from plurality of nucleic acid samples uniquely identifies the nucleic acid molecules from single isolated cell because claim 56 does not specify if first MID sequences from isolated single cell 1 is different from first MID sequences from isolated single cell 2. Similarly the steps of tagging different second MID sequences to the type of nucleic acid molecules from plurality of nucleic acid samples are omitted.

If the intent is to identify the nucleic acid molecules from each of the single isolated cells with unique first MID sequence, it is suggested to include the additional steps, of tagging nucleic acid sample from each of single isolated cell separately with first MID sequence, wherein the first MID sequence is unique to each of the single isolated cell (i.e., first MID sequence from single isolated cell 1 is different from single isolated cell 2).

For claiming the step of second MID sequences are all different from each other, applicant is suggested to recite the steps associated with instant figures 11 and 13, wherein different sized nucleic acid segments from plurality of nucleic acid samples are tagged with different MID sequences, which is consistent with the novelty of the reflex method of the instant invention.

17. Claims 57-64 are also incomplete because they include all the limitations of claim 56, which is considered to be incomplete for the reasons discussed above.

18. Claims 58-62 are indefinite because it is not clear if "the MID sequences" include the first or the second or both MID sequences. Appropriate clarification is required.

Claim Rejections - 35 USC § 102

19. 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.

20. The following is a quotation of the appropriate paragraphs of pre-AIA 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 –
>
> (e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent.

21. The changes made to 35 U.S.C. 102(e) by the American Inventors Protection Act of 1999 (AIPA) and the Intellectual Property and High Technology Technical Amendments Act of 2002 do not apply when the reference is a U.S. patent resulting directly or indirectly from an international application filed before November 29, 2000. Therefore, the prior art date of the reference is determined under 35 U.S.C. 102(e) prior to the amendment by the AIPA (**pre-AIPA** 35 U.S.C. 102(e)).

22. Claims 56-64 are rejected under pre-AIA 35 U.S.C. 102(e) as being anticipated by Linnarsson (WO 2010/117620 published Oct. 14, 2010, priority date Mar. 30, 2009, cited in the IDS of 8/30/17).

Regarding claims 56-58 and 60-61, Linnarsson teaches a method of preparing a nucleic acid libraries (i.e., cDNA libraries) from a plurality of single cells, the method comprising the steps of: releasing mRNA from each single cell to provide a plurality of individual mRNA samples, wherein the mRNA in each individual mRNA (i.e., nucleic acid) sample is from a single cell; synthesizing a first strand of cDNA from the mRNA in each individual mRNA sample and incorporating a tag into the cDNA to provide a plurality of tagged cDNA samples, wherein the cDNA in each tagged cDNA sample is complementary to mRNA from a single cell; pooling the tagged cDNA samples; and amplifying the pooled cDNA samples to generate a cDNA library comprising double-

stranded cDNA, wherein the method further comprises processing the cDNA library to obtain a library suitable for sequencing and sequencing the cDNA library to identify genes originated from the cell source (Figs. 1-4 and their legend, Linnarsson, claims 1-5 and 9-11, the instant steps of claims 56-58 and 60-62 taught by Linnarsson are underlined by the examiner).

The cell specific tag (e.g., see figure 3) in the nucleic acid sample from each of the single cell is the first Multiplex Identifier (first MID) sequences associated with a segment of a nucleic acid molecule of (a) uniquely identifies the single isolated cell from which the nucleic acid molecule is provided.

Linnarsson also teaches that the synthesis of the first strand of cDNA is directed by a cDNA synthesis primer (CDS) that includes an RNA complementary sequence (RCS) that is at least partially complementary to one or more mRNA in an individual mRNA sample (Linnarsson claim 3), wherein a template-switching oligonucleotide (TSO) is added to each individual mRNA sample, wherein said TSO comprises a portion which is at least partially complementary to a portion at the 3' end of the first strand of cDNA (Linnarsson claim 5), wherein the CDS and the TSO each include a different tag (Linnarsson claims 10 and 11). Different tags associated with cDNA segment of Linnarsson meets the limitation of second MID sequences are different from each other.

Regarding claim 59 requiring the MID sequences are appended with ligation, Linnarsson teaches ligating the adaptor to the nucleic acid segments that comprise MID sequences CDS and TSO (Fig. 8 and pg. 29, lines 3-11).

Regarding claim 62 requiring MID sequences are from 2 to 100 bases in length, Linnarsson teaches that the tag sequences (i.e., MID sequences) are from 4-20 nucleotides in length (Linnarsson claim 12), which is within the claimed range.

Regarding claims 63 and 64, Linnarsson teaches that the length is from 200 bp to 5 kb (Fig. 16), which is within the claimed range.

Double Patenting

23. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the claims at issue are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); In re Vogel, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l) (1) - 706.02(l) (3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

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

24. Claims 56-64 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-13 of U.S. Patent No. 9,410,149 in view of Linnarsson (WO 2010/117620 published Oct. 14, 2010, priority date Mar. 30, 2009, cited in the IDS of 8/30/17) for the following reasons.

Regarding instant claim 56, claims 1 and 10 of '149 patent disclose a method of analyzing genomic DNA, comprising: a) generating a first plurality of different segments of a first nucleic acid molecule of a contiguous region in the genomic DNA, wherein said contiguous region in the genomic DNA is represented by a plurality of nucleic acid molecules, and wherein each of said first plurality of different segments is appended to a first multiplex identifier (MID) sequence; b) generating a second plurality of different segments of a second nucleic acid molecule of said contiguous region, wherein each of said second plurality of different segments is appended to a second MID sequence and said first MID sequence and said second MID sequence are different; and c) sequencing the first and second plurality of different segments and the appended first and second MID sequences, respectively, to analyze the genomic DNA, wherein the genomic DNA is genomic DNA from a virus or a bacterium (the steps that are common between instant claim 56 and claims 1 and 10 of '149 patent are underlined by the examiner). One having ordinary skill in the art would recognize that genomic DNA from a bacterium meets the limitation of an isolated single cell.

Claims of '149 patent do not disclose step of providing a plurality of nucleic acid samples each made from single isolated cells and the identifier sequence that uniquely identifies the single isolated cell, which is taught by Linnarsson, who teaches a method of preparing a nucleic acid libraries (i.e., cDNA libraries) from a plurality of single cells,

the method comprising the steps of: releasing mRNA from each single cell to provide a plurality of individual mRNA samples, wherein the mRNA in each individual mRNA sample is from a single cell; synthesizing a first strand of cDNA from the mRNA in each individual mRNA sample and incorporating a tag into the cDNA to provide a plurality of tagged cDNA samples, wherein the cDNA in each tagged cDNA sample is complementary to mRNA from a single cell; pooling the tagged cDNA samples; and amplifying the pooled cDNA samples to generate a cDNA library comprising double-stranded cDNA, wherein the method further comprises processing the cDNA library to obtain a library suitable for sequencing and sequencing the cDNA library to identify genes originated from the cell source (Fig. 1 and its legend, Linnarsson, claims 1-5 and 9-11). Linnarsson also teaches pooling the samples simplifies handling of the samples from each single cell and reduces the time required to analyze gene expression in the single cells, which allows high throughput analysis of gene expression. Pooling the samples prior to amplification also provides the advantage that technical variation between samples is virtually eliminated (pg. 11, paragraph 1), thus providing motivation to include the step of including the tagging the nucleic acid sample for each of the isolated single cell and pooling the plurality of isolated single cell nucleic acids in the claims of '149 patent.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the step of including the tagging the nucleic acid sample for each of the isolated single cell and pooling the plurality of isolated single cell nucleic acids in the claims of '149 patent with a reasonable expectation of success with the expected benefit of allowing high throughput analysis of

gene expression and eliminating the technical variation between samples as taught by Linnarsson (pg. 11, paragraph 1). An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including additional step of tagging nucleic acid sample with identifier sequence which is routinely practiced in the art as exemplified Linnarsson.

Regarding instant dependent claims 57-64, claims 1-13 of '149 patent in view of Linnarsson disclose the steps of said dependent claims and thus are not patentably distinct.

25. Claims 56-64 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-19 of U.S. Patent No. 9,410,150 in view of Linnarsson (WO 2010/117620 as cited above) for the following reasons.

Regarding instant claim 56, claims 1, 10, 12, 13 and 19 of '150 patent disclose the steps of providing a first plurality of different segments of DNA from a first sample, wherein the sample is a single cell, wherein the first plurality of different segments of DNA are derived from a first nucleic acid molecule of a locus in genomic DNA and wherein each of said first plurality of different segments is appended to a first sequence tag; and b) a second plurality of different segments of DNA from the first sample, wherein the second plurality of different segments of DNA are derived from a second nucleic acid molecule of the locus and wherein each of said second plurality of different segments is appended to a second sequence tag and said first sequence tag and said second sequence tag are different, wherein the first plurality of different segments the second plurality of different segments further comprise a multiplex identifier (MID)

sequence that identifies the source of the segments of DNA wherein the MID sequence identifies an individual person, a tissue, a cell . Claim 10 of '150 patent discloses the step of wherein the first plurality of different segments and the second plurality of different segments comprise one or more of an amplification primer sequence and a sequencing primer sequence appended thereto (the steps that are common between instant claim 56 and claims 1, 12, 13 and 19 of '150 patent are underlined by the examiner). One having ordinary skill in the art would recognize that the sequencing of the nucleic acid segments using primer amounts to sequencing the tagged segments of to obtain the nucleotide sequences of the plurality of nucleic acids and the attached first and second MID sequences because the first and second sequence tag amount to two different second MID sequences.

Claims of '150 patent do not disclose step of providing a plurality of nucleic acid samples each made from single isolated cells and analyzing the nucleic acid samples, which is taught by Linnarsson, who teaches a method of preparing a nucleic acid libraries (i.e., cDNA libraries) from a plurality of single cells and sequencing the cDNA library to identify genes originated from the cell source (Fig. 1 and its legend, Linnarsson, claims 1-5 and 9-11). Linnarsson also teaches pooling the samples simplifies handling of the samples from each single cell and reduces the time required to analyze gene expression in the single cells, which allows high throughput analysis of gene expression. Pooling the samples prior to amplification also provides the advantage that technical variation between samples is virtually eliminated (pg. 11, paragraph 1), thus providing motivation to include the step of providing a plurality of nucleic acid

samples each made from single isolated cells and analyzing the nucleic acid samples in the claims of '150 patent.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the step of providing a plurality of nucleic acid samples each made from single isolated cells and analyzing the nucleic acid samples of Linnarsson in the claims of '150 patent with a reasonable expectation of success with the expected benefit of allowing high throughput analysis of gene expression and eliminating the technical variation between samples as taught by Linnarsson (pg. 11, paragraph 1). An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including additional step of tagging nucleic acid sample with identifier sequence which is routinely practiced in the art as exemplified Linnarsson.

Regarding instant dependent claims 57-64 claims 1-19 of '150 patent in view of Linnarsson discloses the steps of said dependent claims and thus are not patentably distinct.

26. Claims 56-64 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-12 of U.S. Patent No. 8,679,756 in view of Linnarsson (WO 2010/117620 as cited above) for the following reasons.

It is noted that the subject matter of claims 1-12 of '756 patent is similar to claims 1-13 of '149 patent. Therefore analysis is very similar. Thus it is maintained that steps of instant claims 56-64 are not significantly different from claims 1-12 of '756 patent in view of Linnarsson.

27. Claims 56-64 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-11 of U.S. Patent No. 8,563,274 in view of Linnarsson (WO 2010/117620 as cited above) for the following reasons.

It is noted that the subject matter of claims 1-11 of '274 patent is similar to claims 1-13 of '149 patent. Therefore analysis is very similar. Thus it is maintained that steps of instant claims 56-64 are not significantly different from claims 1-11 of '274 patent in view of Linnarsson.

Conclusion

28. No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to NARAYAN BHAT whose telephone number is (571)272-5540. The examiner can normally be reached on MON-THURS 8.00 am -6.30pm.

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

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave Nguyen can be reached on 5712720731. 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.

NARAYAN BHAT
Primary Examiner
Art Unit 1634

/NARAYAN BHAT/
Primary Examiner, Art Unit 1634

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14/792,094	Brenner et al.
	Examiner	Art Unit
	NARAYAN BHAT	1634

CPC - Searched*		
Symbol	**Date**	**Examiner**
C12Q 1/68, C12Q 1/6874, C07H 21/02 and C40B 30/04	1/6/16	Bhat N.
Searched uisng key words in different CPC classes. See the search history in EAST.	1/6/16	Bhat N.

CPC Combination Sets - Searched*		
Symbol	**Date**	**Examiner**

US Classification - Searched*			
Class	**Subclass**	**Date**	**Examiner**

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

/NARAYAN BHAT/ Primary Examiner.Art Unit 1634	

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14/792,094	Brenner et al.
	Examiner	Art Unit
	NARAYAN BHAT	1634

Search Notes		
Search Notes	**Date**	**Examiner**
Reviewed the copending applications for the inventors in PLAM and copending claims for potential ODP issues.	1/6/16	Bhat N.
Reviewed the prosecution histories of copending applications resulting in the issuance of patents.	1/6/16	Bhat N.
Reviewed the cited arts in the IDS of 10/9/15.	1/6/16	Bhat N.
searched for the topics in EAST, Pubmed and Google scholar. Search histories are enclosed.	1/6/16	Bhat N.
Updated the search for the topics in EAST. Search history is enclosed.	7/17/16	Bhat N.
C12Q 1/68, C12Q 1/6874, C07H 21/02 and C40B 30/04	1/6/16	Bhat N.
Updated the search for the topics in EAST, Pubmed and Google scholar. Search histories are enclosed.		
Updated the search for the topics in EAST. Search history is enclosed.	5/25/17	Bhat N.
Updated the search for the topics in EAST, Pubmed and Google scholar. Search histories are encloased.	09/13/2017	Bhat N.
Reviewed the arts filed in the IDS (8/30/17)	09/13/2017	Bhat N.

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

/NARAYAN BHAT/ Primary Examiner.Art Unit 1634	

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 01096

Examiner-Initiated Interview Summary	Application No. 14/792,094	Applicant(s) Brenner et al.	
	Examiner NARAYAN BHAT	Art Unit 1634	AIA Status No

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

(1) Examiner: Bhat. (3) _____.

(2) Representative: Ms. Talukder. (4) _____.

Date of Interview: 08 September 2017.

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: 56.

Identification of prior art discussed: None.

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...)

See Continuation Sheet.

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of 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

/NARAYAN BHAT/ Primary Examiner, Art Unit 1634	

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Continuation of Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments:

In view of instant application being part of family of patents, for compact prosecution, the examiner contacted the applicant's representative Ms. Talukder telephonically on 9/8/2017 to inform that further amendments are needed to overcome the potential rejections under 35 USC 112 first and second paragraphs and to fix the minor informalities.

The examiner suggested to further clarifications in claim 56 are needed to specify that the plurality of nucleic acid segments are made from each of the single isolated cell, wherein the plurality of nucleic acid segments from each single cell have cell specific multiplex identifier and different sizes of the nucleic acids from each cell have different multiplex identifier consistent with results schematically presented in figures 11 and 12 (top panel). The examiner also informed that claim 56 as recited is not clear how plurality of nucleic acid samples from plurality of single isolated cell tagged with first and second multiplex identifiers capable identifying origin of nucleic acid sample from each of the single isolated cell.

The amendments to include the step of isolating nucleic acids from each of the isolated single cell separately and appending nucleic acid sample with cell specific identifier were discussed. But no specific amendments to clarify how second MID (multiplex identifier) are different from each other were discussed in view of 35 USC 112 rejections are not being on the record. In view of time constraint to move the instant application on an expedited basis on the part of the examiner and to get specific claim language approval from the applicant, the examiner informed that the office action will be issued to decide further course of action.

No specific claim languages were agreed to during the interview.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/792,094	07/06/2015	Sydney Brenner	104371-5016-US05	9697

43850 7590 05/24/2018

MORGAN, LEWIS & BOCKIUS LLP (SF)
One Market, Spear Street Tower, Suite 2800
San Francisco, CALIFORNIA 94105
UNITED STATES OF AMERICA

EXAMINER	
BHAT, NARAYAN KAMESHWAR	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
05/24/2018	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):

donald.mixon@morganlewis.com
sfipdocketing@morganlewis.com

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01099

Notice of Abandonment	**Application No.** 14/792,094	**Applicant(s)** Brenner et al.
	Examiner NARAYAN BHAT	**Art Unit** 1634

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

This application is abandoned in view of:

1. ☑ Applicant's failure to timely file a proper reply to the Office letter mailed on 20 September 2017.
   (a) ☐ A reply was received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the period for reply (including a total extension of time of _____ month(s)) which expired on _____.
   (b) ☐ A proposed reply was received on _____, but it does not constitute a proper reply under 37 CFR 1.113 to the final rejection. (A proper reply under 37 CFR 1.113 to a final rejection consists only of:(1) a timely filed amendment which places the application in condition for allowance; (2) a timely filed Notice of Appeal (with appeal fee); or (3) if this is utility or plant application, a timely filed Request for Continued Examination (RCE) in compliance with 37 CFR 1.114. Note that RCEs are not permitted in design applications.)
   (c) ☐ A reply was received on _____ but it does not constitute a proper reply, or a bona fide attempt at a proper reply, to the non-final rejection. See 37 CFR 1.85(a) and 1.111. (See explanation in box 7 below).
   (d) ☑ No reply has been received.

2. ☐ Applicant's failure to timely pay the required issue fee and publication fee, if applicable, within the statutory period of three months from the mailing date of the Notice of Allowance (PTOL-85).
   (a) ☐ The issue fee and publication fee, if applicable, was received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the statutory period for payment of the issue fee (and publication fee) set in the Notice of Allowance (PTOL-85).
   (b) ☐ The submitted fee of $ _____ is insufficient. A balance of $ _____ is due.
   The issue fee required by 37CFR 1.18 is $ _____ . The publication fee, if required by 37 CFR 1.18(d), is $ _____ .
   (c) ☐ The issue fee and publication fee, if applicable, has not been received.

3. ☐ Applicant's failure to timely file corrected drawings as required by, and within the three-month period set in, the Notice of Allowability (PTO-37).
   (a) ☐ Proposed corrected drawings were received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the period for reply.
   (b) ☐ No corrected drawings have been received.

4. ☐ The letter of express abandonment which is signed by the attorney or agent of record or other party authorized under 37 CFR 1.33 (b). See 37 CFR 1.138(b).

5. ☐ The letter of express abandonment which is signed by an attorney or agent (acting in a representative capacity under 37 CFR 1.34) upon the filing of a continuing application.

6. ☐ The decision by the Board of Patent Appeals and Interference rendered on _____ and because the period for seeking court review of the decision has expired and there are no allowed claims.

7. ☑ The reason(s) below:

As discussed in the interview summary, applicant's representative Ms. Talukder did not respond to the message left on her voice mail on 4/18/18 by 4/21/18 and therefore the notice of abandonment has been issued.

/NARAYAN K BHAT/
Primary Examiner, Art Unit 1634

Petitions to revive under 37 CFR 1.137, or requests to withdraw the holding of abandonment under 37 CFR 1.181, should be promptly filed to minimize any negative effects on patent term.

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01100

Examiner-Initiated Interview Summary	Application No. 14/792,094	Applicant(s) Brenner et al.	
	Examiner NARAYAN BHAT	Art Unit 1634	AIA Status No

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

(1) Examiner: Bhat. (3) _____.

(2) Representative: Ms. Talukder. (4) _____.

Date of Interview: 18 April 2018.

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: 56.

Identification of prior art discussed: Arts of the record.

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...)

Foe compact prosecution the examiner contacted the applicant's representative, Ms. Talukder and a left a message on her voice mail on 4/18/18 to inquire if any response has been filed to the Non-final rejection mailed on 9/20/2017 and informed that if examiner did not get a reply by 4/21/18, the examiner will send a notice of abandonment. No response has been filed till 5/19/2018..

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of 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

	/NARAYAN K BHAT/ Primary Examiner, Art Unit 1634

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 01101

PTO/SB/122 (04-15)
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Application

Address to:
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Application Number	14/792,094
Filing Date	July 6, 2015
First Named Inventor	SYDNEY BRENNER
Art Unit	1634
Examiner Name	Narayan Kameshwar Bhat
Attorney Docket Number	104371-5016-US05

Please change the Correspondence Address for the above-identified patent application to:

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I am the:

☐ Applicant/Inventor

☐ Assignee of record of the entire interest.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

☒ Attorney or agent of record. Registration Number 61,368

☐ Registered practitioner named in the application transmittal letter in an application without an executed oath or declaration. See 37 CFR 1.33(a)(1). Registration Number_____.

Signature	/Gargi Talukder/
Typed or Printed Name	Gargi Talukder
Date	August 10, 2018
Telephone	415.442.1000

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☐ *Total of _____forms are submitted.

This collection of information is required by 37 CFR 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 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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Electronic Acknowledgement Receipt	
EFS ID:	33437777
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	43850
Filer:	Gargi Talukder/Sheila Gloss
Filer Authorized By:	Gargi Talukder
Attorney Docket Number:	104371-5016-US05
Receipt Date:	10-AUG-2018
Filing Date:	06-JUL-2015
Time Stamp:	17:21:37
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Change of Address	104371-5016-US05_Corr.pdf	65141 9fd3c7e27e9876dc3a2ecc8d6c8e43aeeba7855b71	no	1
Warnings:					

Information:	
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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
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If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.