United States Patent and Trademark Office

Patent Trial and Appeal Board

A petition has been filed in Patent Number 12,168,797, Application Number 18/352,112 on 9/15/25 (Date).

The Case Number is IPR2025-01546.
(IPR, CBM, PGR, DER #)

To view the documents filed in this petition, go to https://ptab.uspto.gov.

Use the Search PTAB tab and enter the Patent Number or the Trial or Case Number and select the Search button.

Questions regarding this notice should be directed to the Patent Trial and Appeal Board at 571-272-7822.

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.
18/352,112	07/13/2023	Emil P. KARTALOV	88DG-373535-US	3145

30764 7590 12/17/2024

Sheppard, Mullin, Richter & Hampton LLP
650 Town Center Drive, 10th Floor
Costa Mesa, CA 92626

EXAMINER	
NEGIN, RUSSELL SCOTT	

ART UNIT	PAPER NUMBER
1686	

NOTIFICATION DATE	DELIVERY MODE
12/17/2024	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):

SheppardMullin_Pair@firsttofile.com
docketing@sheppardmullin.com

APPLICATION NO.	ISSUE DATE	PATENT NO.
18/352,112	17-Dec-2024	12168797

Sheppard, Mullin, Richter & Hampton LLP
650 Town Center Drive, 10th Floor
Costa Mesa, CA 92626

EGRANT NOTIFICATION

Your electronic patent grant (eGrant) is now available, which can be accessed via Patent Center at https://patentcenter.uspto.gov

The electronic patent grant is the official patent grant under 35 U.S.C. 153. For more information, please visit https://www.uspto.gov/electronicgrants

TO: docketing@sheppardmullin.com,SheppardMullin_Pair@firsttofile.com
FROM: noreply@uspto.gov
CC: patentcenter_eofficeaction@uspto.gov
SUBJECT: USPTO: Patent Electronic System - eGrant Notification for Customer Number 30764

Tue Dec 17 06:00:28 EST 2024

Dear Patent Center Customer:

Correspondence Address:

Sheppard, Mullin, Richter &Hampton LLP
650 Town Center Drive, 10th Floor
Costa Mesa,CALIFORNIA,92626
UNITED STATES

This is a courtesy notification regarding the following USPTO patent application(s) associated with your Customer Number, 30764, that have new eGrant(s) and eCofC(s). The eGrant and eCofC are now available for viewing in Patent Center.

The official date of the eGrant notification will be indicated on the form (e.g., PTOL-90) accompanying the correspondence.

Disclaimer:
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Application	Patent	Document	Mailroom Date	Attorney Docket No.
18352112	12168797	EGRANT.NTF	12/17/2024	88DG-373535-US

To examine your eGrant and eCofC online, please access Patent Center then search using the listed application or patent number. To download your eGrant, click on the eGrant and eCofC link on the left-hand menu or select the “Download eGrant” link on the Application data page. To download your eCofC, click on the eGrant and eCofC link on the left-hand menu.

If you have any questions, please contact the Patent Electronic Business Center (EBC) at ebc@uspto.gov or 866-217-9197 Monday – Friday, 6 a.m. to midnight ET.

Please do not reply to this email as it was sent from an unmonitored mailbox.

Sincerely,

The Patent Center Team

TO: docketing@sheppardmullin.com,SheppardMullin_Pair@firsttofile.com
FROM: noreply@uspto.gov
CC: patentcenter_eofficeaction@uspto.gov
SUBJECT: USPTO: Patent Electronic System - Correspondence Notification for Customer Number 30764

Thu Nov 28 05:00:39 EST 2024

Dear Patent Center Customer:

Correspondence Address:

Sheppard, Mullin, Richter &Hampton LLP
650 Town Center Drive, 10th Floor
Costa Mesa,CALIFORNIA,92626
UNITED STATES

This is a courtesy notification regarding the following USPTO patent application(s) associated with your Customer Number, 30764, that have new outgoing correspondence. This correspondence is now available for viewing in Patent Center.

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Application	Document	Mailroom Date	Attorney Docket No.
18352112	ISSUE.NTF	11/27/2024	88DG-373535-US

To view your correspondence online, please sign in to Patent Center and then select Workbench/View correspondence. To update your email address(es), select Manage/Manage customer numbers.

If you have any questions, please contact the Patent Electronic Business Center (EBC) at ebc@uspto.gov or 866-217-9197 Monday – Friday, 6 a.m. to midnight ET.

Please do not reply to this email as it was sent from an unmonitored mailbox.

Sincerely,

The Patent Center Team

UNITED STATES PATENT AND TRADEMARK OFFICE

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Alexandria, Virginia 22313-1450
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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/352,112	12/17/2024	12168797	88DG-373535-US	3145

30764 7590 11/27/2024

Sheppard, Mullin, Richter & Hampton LLP
650 Town Center Drive, 10th Floor
Costa Mesa, CA 92626

ISSUE NOTIFICATION

The projected patent number and issue date are specified above. The patent will issue electronically. The electronically issued patent is the official patent grant pursuant to 35 U.S.C. § 153. The patent may be accessed on or after the issue date through Patent Center at https://patentcenter.uspto.gov/. The patent will be available in both the public and the private sides of Patent Center. Further assistance in electronically accessing the patent, or about Patent Center, is available by calling the Patent Electronic Business Center at 1-888-217-9197.

The USPTO is implementing electronic patent issuance with a transition period, during which period the USPTO will mail a ceremonial paper copy of the electronic patent grant to the correspondence address of record. Additional copies of the patent (i.e., certified and presentation copies) may be ordered for a fee from the USPTO's Certified Copy Center at https://certifiedcopycenter.uspto.gov/index.html. The Certified Copy Center may be reached at (800)972-6382.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Center (https://patentcenter.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Patents Stakeholder Experience (OPSE), Stakeholder Support Division (SSD) at (571)-272-4200.

INVENTOR(s) (Please see PATENT CENTER site https://patentcenter.uspto.gov for additional inventors):

Emil P. KARTALOV, PASADENA, CA;
Aditya RAJAGOPAL, ORANGE, CA;
Axel SCHERER, BARNARD, VT;

APPLICANT(s) (Please see PATENT CENTER site https://patentcenter.uspto.gov for additional applicants):

CALIFORNIA INSTITUTE OF TECHNOLOGY, Pasadena, CA;

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The USA offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to encourage and facilitate business investment. To learn more about why the USA is the best country in the world to develop technology, manufacture products, and grow your business, visit SelectUSA.gov.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/352,112	07/13/2023	Emil P. KARTALOV	88DG-373535-US	3145

30764 7590 11/18/2024

Sheppard, Mullin, Richter & Hampton LLP
650 Town Center Drive, 10th Floor
Costa Mesa, CA 92626

EXAMINER	
NEGIN, RUSSELL SCOTT	

ART UNIT	PAPER NUMBER
1686	

NOTIFICATION DATE	DELIVERY MODE
11/18/2024	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):

SheppardMullin_Pair@firsttofile.com
docketing@sheppardmullin.com

Corrected ***Notice of Allowability***	**Application No.** 18/352,112	**Applicant(s)** KARTALOV et al.	
	Examiner RUSSELL S NEGIN	**Art Unit** 1686	**AIA (FITF) Status** No

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

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to the communications of 11/12/2024.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

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

3. ☑ The allowed claim(s) is/are 1-30 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

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

   Certified copies:

   a) ☐ All b) ☐ Some* c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☐ Examiner's Amendment/Comment
6. ☐ Examiner's Statement of Reasons for Allowance
7. ☑ Other PTO-271 form.

/RUSSELL S NEGIN/
Primary Examiner, Art Unit 1686

IPR2025-01546
California Institute of Technology - Ex. 2026, Page 9

Response to Rule 312 Communication	Application No.	Applicant(s)	
	18/352,112	KARTALOV et al.	
	Examiner	Art Unit	AIA (FITF) Status
	RUSSELL S NEGIN	1686	No

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

1. ☑ The amendment filed on 12 November 2024 under 37 CFR 1.312 has been considered, and has been:

a) ☐ entered.

b) ☑ entered as directed to matters of form not affecting the scope of the invention.

c) ☐ disapproved because the amendment was filed after the payment of the issue fee.

Any amendment filed after the date the issue fee is paid must be accompanied by a petition under 37 CFR 1.313(c)(1) and the required fee to withdraw the application from issue.

d) ☐ disapproved. See explanation below.

e) ☐ entered in part. See explanation below.

f) ☐ not entered because the supplemental or corrected Application Data sheet (ADS)

☐ was not accompanied by a petition to accept an unintentionally delayed claim under 37 CFR 1.55 or 27 CFR 1.78;

☐ did not identify the information being changed in accordance with 37 CFR 1.76(c)(2);

☐ was not properly signed in accordance with 37 CFR 1.76(e) (or 37 CFR 1.33(b) for applications filed prior to September 16, 2012).

/RUSSELL S NEGIN/
Primary Examiner, Art Unit 1686

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

PTO/SB/08a (01-10)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	18352112
Filing Date	2023-07-13
First Named Inventor	Emil P. KARTALOV
Art Unit	1686
Examiner Name	Russell Scott NEGIN
Attorney Docket Number	88DG-373535-US

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2]	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
/RSN/	1	102559868	CN	A	2012-07-11	Univ. Xiamen		☒
/RSN/	2	2004/099434	WO	A2	2004-11-18	The Public Health Res Inst of the City of New York		☐

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/RUSSELL S NEGIN/ 11/14/2024

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	18352112
	Filing Date	2023-07-13
	First Named Inventor	Emil P. KARTALOV
	Art Unit	1686
	Examiner Name	Russell Scott NEGIN
	Attorney Docket Number	88DG-373535-US

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
/RSN/	1	Supplemental Search Report dated October 12, 2024, issued in related Chinese Patent Application No. 202210389347.4 (2 pages).	☐

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

EXAMINER SIGNATURE

Examiner Signature	/RUSSELL S NEGIN/	Date Considered	11/14/2024

*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.

PATENT
Docket No.: 88DG-373535-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	)	
	)	Confirmation No.: 3145
Emil P. KARTALOV *et al.*	)	
	)	Examiner: Russell S. NEGIN
Application No.: 18/352,112	)	
	)	
Filed: July 13, 2023	)	Art Unit: 1672
	)	
For: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	)	***VIA Patent Center***
	)	

OK TO ENTER
/RSN/ 11/14/2024

MAIL STOP ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AFTER ALLOWANCE UNDER 37 C.F.R. § 1.312

Dear Commissioner,

In response to the Notice to File Corrected Application Papers mailed October 30, 2024, the period for response ending on December 30, 2024, Applicant respectfully requests entry of this amendment under 37 C.F.R. 1.312 for the above-captioned patent application prior to issuance of the patent. A Replacement Drawing is also submitted under 37 C.F.R. 1.312 in connection with this Response.

Amendment to the Specification begin on page **2** of this paper.

Remarks begin on page **2** of this paper.

AMENDMENTS TO THE SPECIFICATION

Applicant submits herewith a Substitute Specification (marked and clean) in compliance with 37 C.F.R 1.121. The specification is amended at the following paragraphs to clarify **Fig. 5**:

Paragraph **[0051]**: has been amended to replace "Fig. 5 shows a schematic" with "Figs. 5A and 5B show schematics…"

Paragraphs **[00156]-[00157]** have been amended to rename **Fig. 5**, which shows the two examples of instrumental configurations to **Fig. 5A**, which references **501**, and **Fig. 5B**, which references **507**. Reference names **501** and **507** have been deleted in the Specification to reflect the Replacement Drawing showing the new **Figs. 5A and 5B** do not make reference to **501** and **507**.

No new matter has been introduced by this amendment.

REMARKS

Applicant respectfully requests entry of the clarifying amendments. The amendments do not change the scope of the claims and no new matter has been added.

IPR2025-01546
California Institute of Technology - Ex. 2026, Page 14

CONCLUSION

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. Prompt and favorable consideration of this Response is respectfully requested. A favorable response is awaited. It is believed that no fees are due in connection with this Rule 312 amendment. The Commissioner is authorized to charge any underpayment or credit any overpayment to Deposit account No. 50-4561 (Attorney Docket No. 88DG-373535-US)

Respectfully submitted,

SHEPPARD MULLIN RICHTER & HAMPTON LLP

By____/ David E. Heisey /_____
David E. Heisey
Registration No.: 42,651

Dated: November 12, 2024

12275 El Camino Real, Suite 100
San Diego, CA 92130-4092
Telephone: (858) 720-8900
Facsimile: (858) 509-3691

Customer No. 30764

IPR2025-01546
California Institute of Technology - Ex. 2026, Page 15

TO: docketing@sheppardmullin.com,SheppardMullin_Pair@firsttofile.com
FROM: noreply@uspto.gov
CC: patentcenter_eofficeaction@uspto.gov
SUBJECT: USPTO: Patent Electronic System - Correspondence Notification for Customer Number 30764

Mon Nov 18 05:01:08 EST 2024

Dear Patent Center Customer:

Correspondence Address:

Sheppard, Mullin, Richter &Hampton LLP
650 Town Center Drive, 10th Floor
Costa Mesa,CALIFORNIA,92626
UNITED STATES

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Application	Document	Mailroom Date	Attorney Docket No.
18352112	NOA	11/18/2024	88DG-373535-US
18352112	N271	11/18/2024	88DG-373535-US
18352112	1449	11/18/2024	88DG-373535-US

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If you have any questions, please contact the Patent Electronic Business Center (EBC) at ebc@uspto.gov or 866-217-9197 Monday – Friday, 6 a.m. to midnight ET.

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Sincerely,

The Patent Center Team

PTO/SB/08a (01-10)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99)**		
	Application Number	18352112
	Filing Date	2023-07-13
	First Named Inventor	Emil P. KARTALOV
	Art Unit	1686
	Examiner Name	Russell Scott NEGIN
	Attorney Docket Number	88DG-373535-US

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	102559868	CN	A	2012-07-11	Univ. Xiamen		☒
	2	2004/099434	WO	A2	2004-11-18	The Public Health Res Inst of the City of New York		☐

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Art Unit	1686
	Examiner Name	Russell Scott NEGIN
	Attorney Docket Number	88DG-373535-US

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1	Supplemental Search Report dated October 12, 2024, issued in related Chinese Patent Application No. 202210389347.4 (2 pages).	☐

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

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

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

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

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	Filing Date	2023-07-13
	First Named Inventor	Emil P. KARTALOV
	Art Unit	1686
	Examiner Name	Russell Scott NEGIN
	Attorney Docket Number	88DG-373535-US

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	/David E. Heisey/	Date (YYYY-MM-DD)	2024-11-12
Name/Print	David E. Heisey	Registration Number	42651

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APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
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Title of Invention

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	3145	FILED BY	Annie Wong
PATENT CENTER #	67939458	AUTHORIZED BY	David Heisey
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(54) Title: HOMOGENEOUS MULTIPLEX SCREENING ASSAYS AND KITS

(57) Abstract: Highly multiplexed homogeneous *in vitro* screening assays for numerous possible nucleic acid targets, any of which might be present in a sample, utilize fluorescent hybridization probes that are combinatorially coded from a panel of fluorophores by subdividing each probe into portions and differently labeling each portion such that, when portions are combined, each probe has a unique code. The assays may include target amplification and real-time detection. Probe sets and kits containing additional assay reagents may be used to perform the screening assays.

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HOMOGENEOUS MULTIPLEX SCREENING ASSAYS AND KITS

TECHNICAL FIELD

This invention relates to homogenous *in vitro* multiplexed assays, including particularly homogeneous amplification assays, for screening biological samples for the presence of any of a large number of nucleic acid sequences.

BACKGROUND

A variety of assay techniques are available for testing a biological sample obtained from any of a variety of sources for the presence of a nucleic acid sequence that may indicate the presence, for example, of a particular bacterium, virus or other pathogen, including a particular strain or mutant. Assays are also available for testing such samples for the presence of a nucleic acid sequence of the subject's own genomic DNA that may indicate the presence, for example, of one or another disease-related genetic mutation. Assays may include oligonucleotide probes bearing detectable labels, for example, P^{32} or fluorophores. Nucleic acids, either DNA or RNA, in a sample may be probed directly. Alternatively, assays may include amplification of target sequences by any of several amplification techniques, for example, PCR, NASBA or TMA. Amplification assays may be monitored in real time utilizing intercalating dyes, for example SYBR green, or fluorescently labeled probes, such as 5' nuclease probes Livak, K.J. *et al.* (1995), Oligonucleotides with fluorescent Dyes at Opposite Ends Provide a Quenched Probe System Useful for Detecting PCR Product and Nucleic Acid Hybridization, PCR Meth. Appl. 4: 357-362, dual FRET probes, Espy, M.J. *et al.* (2002), Detection of Vaccinia Virus, Herpes Simplex Virus, Varicella-Zoster Virus, and *Bacillus anthracis* by LightCycler Polymerase Chain Reaction after Autoclaving: Implications for Biosafety of Bioterrorism Agents, Mayo Clin. Proc. 77: 624-628, or molecular beacon probes, Tyagi, S. and Kramer, F.R. (1996), Molecular Beacons: Probes that Fluoresce upon Hybridization, Nature Biotechnol. 14: 303-308; Tyagi, S. *et al.* (1998), Multicolor Molecular Beacons for Allele Discrimination, Nature Biotechnol. 16: 49-53. Real-time multiplex assays utilizing PCR amplification have been demonstrated with TaqMan dual-labeled linear

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probes and the 5' nuclease detection process and, alternatively, with PCR amplification and molecular beacon probes, Tyagi. S. *et al.* (1998), *supra*; Vet, J.A. *et al.* (1999), Multiplex Detection of Four Pathogenic Retroviruses Using Molecular Beacons, Proc. Natl. Acad. Sci. USA 96: 6394-6399; El-Hajj, H. *et al.* (2001), Detection of Rifampin Resistance in *Mycobacterium tuberculosis* in a Single Tube with Molecular Beacons, J. Clin. Microbiol. 39: 4131-4137. Fluorescence-based multiplex assays currently are limited to about eight targets per sample by the need to minimize overlaps in emission spectra of fluorophores and, hence, are not expandable for use as highly multiplexed screening assays.

Highly multiplexed assays rely on spatial segregation of targets for signal resolution. Spatial segregation enables the use of coding schemes involving combinations of differently colored fluorophores (combinatorial coding), combinations of different amounts of each fluorophore (ratio coding), and both. One example of an assay with spatial segregation is fluorescence *in situ* hybridization, or FISH, for chromosomal analysis. Speicher *et al.* (1996), Karyotyping Human Chromosomes by Combinatorial Multi-fluor FISH, Nature Genet. 12: 368-375, for example, report the use of 27 probes combinatorially labeled using a set of six different fluorophores for analysis of chromosome spreads. Similarly, segregation of transcription sites in cell nuclei has enabled combinatorial coding utilizing multiple, singly labeled probes per site, as well as ratio coding. Singer, R.H., International (PCT) patent application WO 00/65094; Levsky, J.M. *et al.* (2002), Single-Cell Gene Expression Profiling, Science 297: 836-840. Another spatial-segregation probe technique is the use of multiplex probe arrays, including arrays on DNA chips. Schena, M. *et al.* (1995), Quantitative Monitoring of Gene Expression Patterns with a Complementary DNA Microarray, Science 20: 467-470; Gingeras, T.R. *et al.* (1998), Simultaneous Genotyping and Species Identification Using Hybridization Pattern Recognition Analysis of Generic *Mycobacterium* DNA Arrays. Genet. Res. 8: 435-448; Han *et al.* (2001), Quantum-Dot-Tagged Microbeads for Multiplexed Optical Coding of Biomolecules, Nature Biotechnol. 19, 631-635. Another segregation approach is the use if electrophoresis to separate ligated probe pairs of differing lengths. Tong, A.K. *et al.* (2001), Combinatorial Fluorescence Energy

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Transfer Tags for Multiplex Biological Assays, Nature Biotechnol. 19: 756-759. In that method, a variation of the oligonucleotide ligation assay ("OLA") for SNP detection, differently labeled probes are ligated to capture probes on a target, and the hybrids are immobilized, washed, released, separated from one another by electrophoresis, and read for each probes' fluorescent code, which is a combination of colors and ratios. Array methods and electrophoretic methods remain technically complex, requiring many separate steps, including amplification, hybridization, washing and analysis.

There is not currently available a homogeneous fluorescence hybridization assay that is suitable for use as a highly multiplexed screening assay, despite the need for such an assay. During suppressive treatment of the HIV-1 virus with protease inhibitors, for example, any of about 30 mutations is likely to proliferate over time and to require change in treatment. Hirsch, M.S. *et al.* (1998), Antiretroviral Drug Resistance testing in Adults with HIV Infection, JAMA 279: 1984-1991. Lacking a highly multiplexed screening assay, current practice is to sequence the virus in response to a patient's increase in viral load. Sequencing is made difficult by the fact that the arising mutant is not the only allele present. During initial diagnosis of a patient with particular symptoms, for example fever, there is available no highly multiplexed homogeneous assay to screen for an early indication of one of numerous possible infectious agents that may be the cause of the patient's symptoms.

An aspect of this invention is highly multiplexed homogeneous assays for screening samples for the presence of a target nucleic acid sequence from among at least ten, and as many as 60 or more, possible targets, utilizing conventional fluorescence detection equipment and techniques, and fluorescently labeled hybridization probes.

Another aspect of this invention is such screening assays that employ target amplification, optionally with real-time detection, that are capable of detecting small amounts of pathogens that may be found in otherwise sterile samples such as blood.

Yet another aspect of this invention is kits and oligonucleotide sets for carrying out particular screening assays according to this invention.

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SUMMARY

Assays according to this invention are highly multiplexed assays suitable for screening. By "highly multiplexed," I mean that an assay is capable of detecting any of at least six, preferably at least 10, and in certain embodiments 30-60 and even more possible target sequences.

Assays according to this invention are homogeneous nucleic acid *in vitro* assays fluorescently labeled utilizing hybridization probes. By "homogeneous", I mean that nucleic acid targets are detected in solution without segregating targets spatially and without washing away unbound hybridization probes. Fluorescence signal is obtained from the reaction mixture itself. Certain preferred embodiments include target amplification by, for example PCR or NASBA, which enables detection of small amounts of pathogens in samples. When amplification is used, the reaction vessel, for example a microcentrifuge tube, is hermetically sealed to prevent contamination of the other samples being tested at the same time, samples not yet tested, equipment and workers.

Detection in assays according to this invention is by fluorescently labeled nucleic acid hybridization probes. Fluorescent labels, when stimulated emit radiation in the visible, ultraviolet or near infrared portions of the electromagnetic spectrum. Each probe is specific to one target among the multitude of possible targets being screened. If the goal and design of an assay are to distinguish among bacterial species, one will design a probe for a species, for example against a conserved region of *M. tuberculosis*, or design a probe that hybridizes to non-identical sequences of multiple strains of the same species. If the goal, on the other hand, is to distinguish among strains or mutant alleles, one will design each probe against a variable region to provide the necessary distinction and make the probe strain-specific. Preferred probes for use in this invention are molecular beacon probes, which may be made mismatch tolerant or mismatch intolerant, depending on the need of a particular assay. As is known in the art, choice of target sequence and probe design are balanced to minimize both false negatives and false positives.

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Multiplex capability is achieved by combinatorial coding, with or preferably without added ratio coding, utilizing a panel of distinguishable fluorophores. In a combinatorial code, each code element is identified by its unique pattern of different labels, in this case colors. The combinatorial code includes up to four different fluorophores per code element. The different fluorophores are from a panel of at least four distinguishable fluorophores, more preferably a panel of 4-8 distinguishable fluorophores. The combinatorial code may be duplex coding in which the code elements comprise two colors, optionally enlarged by additional code elements of one color. The combinatorial code may by triplex coding in which the code elements comprise three colors, optionally enlarged by additional code elements of two colors (triplex code combined with a duplex code), and optionally enlarged by code elements of one color. The combinatorial code may be quadraplex coding in which the code elements comprise four colors, optionally enlarged by additional code elements of three colors, two colors or one color, or some combination thereof.

Rather than labeling individual probe molecules with multiplex colors to give the probe the pattern assigned to it as a code element, each probe is subdivided into the required number of portions, and each portion is labeled to emit a different color signal. The portions are then mixed to create a probe that, when hybridized to its target, will include the unique color signal pattern of the code element.

For the preferred highly quenched probes such as molecular beacon probes, each portion will emit a single color. For less preferred probes labeled with two different interacting fluorophores, such as 5'-nuclease probes, one option is to utilize a common short-wavelength fluorophore (which I call the "harvester" fluorophore), vary the long-wavelength fluorophores (which I call the "emitter" fluorophores), and detect the signal change in the latter. In that case, each portion will emit a signal in a different color. Another option is the reverse, namely, to use a common emitter fluorophore, vary the harvester fluorophores, and detect the signal change in the latter. In that case, each portion will also emit a signal in a different color, but for purposes of coding it is the harvester that becomes the detected emitter. However, a third option is to vary both fluorophores to permit detection of a ratio change in their emissions, so that the signal from one portion might be the ration a/b, the ratio from a

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second portion might be the ratio c/d, and the ratio from a third portion might be e/f, where a, b, c, d, e and f are distinguishable fluorophores. In this case the long-wavelength fluorophores are varied and can be considered to characterize the signal one obtains from any given probe portion. For ease of understanding, descriptions of combinatorial codes in this application and in the figures generally describe embodiments in which one detects for each probe portion the emission of a particular color, which can be characterized as the emitter fluorophore. It is to be understood that the teachings also apply to embodiments utilizing ratios, and persons skilled in the art will be able to apply the teachings to those embodiments.

If one begins with a panel of eight different fluorophores, for example, and chooses to subdivide each probe into four portions and to label each of the four portions with a different color, the codebook can include as many as 70 different combinations; if one begins with the same-sized panel of fluorophores but subdivides each probe into three portions and labels each of the three portions with a different color, the codebook can include as many as 56 different combinations; and if one begins with the same-sized panel of fluorophores but subdivides each probe into two portions and labels each of the two portions with a different color, the codebook can include as many as 28 different combinations. Four-color, three-color and two-color codes may be combined with each other and even with a one-color code, but my most preferred embodiments do not include such combinations. In ratio coding differing amounts of the same label are used for differentiation. Combinatorial codes according to this invention may be augmented with ratio coding, preferably integral ratio coding, that is, ratios of whole numbers such as 2:1, 3:1, 4:1 or 5:1, and vice versa. It is preferred that portions of molecular beacon probes be labeled with a non-fluorescent quencher and one signature-color fluorophore, most preferably without added ratio coding for reasons that will be explained.

As stated above, assays according to this invention are homogeneous detection assays. There is no segregation of targets. There is no separation of unbound probes from probes bound to their targets. Consequently, in assays according to this invention there must be a detectable signal change indicative of a probe hybridizing to its target. Preferred molecular beacon probes are highly quenched when free-floating

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at the detection temperature in the assay but change conformation and fluoresce when hybridized to their respective targets. Another type of probe whose fluorescence is restored by hybridization to its target and that is generally suitable for use in assays according to this invention is a "yin-yang" probe, a bimolecular probe labeled with a fluorophore on a target complementary strand and a quencher on a competing strand. Li, Q. *et al.* (2002), A New Class of Homogeneous Nucleic Acid Probes Based on Specific Displacement Hybridization, Nucleic Acids Res. 30: e5. Other probe-assay schemes result in a fluorescent signal change indicative of probes hybridizing to targets. In the 5'-nuclease detection process, end-labeled linear (or random-coil) probes with two different fluorophores interact by fluorescence resonance energy transfer (FRET) when free-floating but are cleaved during primer extension in a polymerase chain reaction (PCR) amplification, resulting in a signal change (emission from the shorter wavelength fluorophore increases, emission from the longer wavelength fluorophore decreases, and the ratio of the two changes accordingly). Livak, K.J. *et al.* (1995), *supra*. Another system, so-called LightCycler probes, works in the opposite manner. Each probe is a pair of fluorescently labeled linear oligonucleotides that hybridize adjacently on a target and interact by FRET, thereby producing a signal change (emission from the shorter wavelength fluorophore decreases, emission from the longer wavelength fluorophore increases, and the ratio of the two changes accordingly). Espy, M.J. *et al.* (2002), *supra*. For FRET systems, I refer to the shorter wavelength fluorophore as a "harvester" and the longer wavelength fluorophore as an "emitter". However, because the background resulting from FRET quenching is considerably higher than contact-mediated quenching that is commonly used in molecular beacon probes and yin-yang probes (Tyagi *et al.* (1998), *supra*; Marras, S.A.E. *et al.* (2002), Efficiencies of Fluorescence Resonance Energy Transfer and Contact-Mediated Quenching in Oligonucleotide Probes, Nucleic Acids Res. 30: e122), FRET quenching is not preferred for screening assays according to this invention. Assays according to this invention preferably employ probes that are quenched by contact-mediated quenching, such as molecular beacon probes or yin-yang probes. My most preferred probes are molecular beacon probes that include a non-fluorescent quencher. These probes are most preferred, not only because of

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their low background signal, but also because of their ease of design for a multiplex system. Bonnet, G. *et al.* (1998), Thermodynamic Basis of the Enhanced Specificity of Structured DNA Probes, Proc. Natl. Acad. Sci. USA 96: 6171-6176.

Assays according to this invention include, but are not limited to, embodiments that employ target amplification. Several exponential amplification techniques are well known. Among them are the polymerase chain reaction (PCR), which includes thermal cycling, and isothermal amplification schemes such as nucleic acid sequence-based amplification (NASBA), strand-displacement amplification (SDA), transcription-mediated amplification (TMA), rolling-circle amplification (RCA), and ramification amplification methodology (RAM). Amplification assay embodiments may utilize end-point detection or real-time detection. It is preferred to perform amplification in a sealed environment, for example sealed tubes, in the presence of the hybridization probes to minimize the possibility of cross contamination between samples that results from opening sample tubes.

While not limited by sample source, assays according to this invention advantageously may utilize human or animal samples sometimes referred to as being normally sterile, such as blood, tissue or spinal fluid. Such samples are advantageous as compared, for example, to sputum, stool or environmental samples, because they will contain fewer extraneous DNA sequences that could potentially interfere with amplification and detection. It is known that at least small amounts of many pathogens can be found in a patient's blood, even in septic syndromes resulting from bacterial infections. It is preferred to utilize normally sterile sources rather than non-sterile sources where a particular screening assay permits a choice.

Certain amplification assays according to this invention may advantageously take advantage of primers that amplify all or a number of nucleic acid targets being screened. When screening for mutations, it may be that numerous possible mutations occur in a particular variable nucleic acid sequence of interest, and one pair of primers flanking that region can be used to amplify all such possible mutations. Certain of these so-called "universal primers" bind to conserved regions of multiple species and are used for amplifying numerous targets, for example genes of various bacteria. See,

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for example, Kox, L.F.F. *et al.* (1995), PCR Assay Based on DNA Coding for 16S rRNA for Detection and Identification of Mycobacteria in Clinical Samples. J. Clin. Microbiol. 33: 3225-3233; Iwen, P.C. *et al.* (1995), Evaluation of Nucleic Acid-Based Test (PACE 2C) for Simultaneous detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in Endocervical Specimens, J. Clin. Microbiol. 33: 2587-2591; Yang, S. *et al.* (2002), Quantitative Multiprobe PCR Assay for Simultaneous Detection and Identification to Species Level of Bacterial Pathogens, J. Clin. Microbiol. 40: 3449-3454; Schonhuber, W. *et al.* (2001), Utilization of mRNA Sequences for Bacterial Identification, BMC Microbiol. 1: 2; Wong, R.S. and Chow, A.W. (2002), Identification of Enteric Pathogens by Heat Shock Protein 60kDa (HSP 60) Gene Sequences, FEMS Microbiol. Lett. 206: 107-113. One pair, more likely two or three pairs, of primers may suffice for particular screening assays.

This invention includes assay kits for performing particular multiplex screening assays according to this invention. Kits include at least the intended complement of detection probes. We refer to collections of oligonucleotides as "oligonucleotide sets." When the assay is an amplification assay, for example a PCR assay, the oligonucleotide sets preferably also include the primers needed for amplification. They may also include any amplifiable control sequence. Kits may include additional assay reagents such as enzymes, amplification buffer and, if desired, sample preparation reagents for isolation of nucleic acids; that is, up to all reagents needed for performing the assay, from sample preparation through detection.

Fluorescence-based screening assays of this invention are generally adaptable to detection instruments, which may include thermal cycling capability. Instruments may use a single-wavelength source or a multi-wavelength source, for example a white light combined with selectable filters, multiple laser sources or multiple light-emitting diode sources. Detection instruments vary in their ability to distinguish emissions from multiple fluorophores, which needs to be taken into account when selecting fluorophore panels for a particular assay. In preferred embodiments of assays according to this invention, emissions from fluorophores for each probe are balanced in intensity such that intensities from the subdivided portions are closely spaced, which aids identification. Fluorophores vary in intensity, target sequences

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vary in accessibility for probe hybridization, and instruments may vary in their responses to different wavelengths. Relative amounts of portions may be varied to achieve balance. Where possible, it is preferred that all portions for all probes be balanced to further aid in identification. The results of these assays can be automatically decoded and interpreted by computer programs in real time, enabling the assays to be highly accurate, and to be carried out in an automated, high-throughput fashion in clinical diagnostic settings.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1 is a table showing the numbers of distinguishable combinations obtained using two or three colors per probe and panels of 4-8 distinguishable fluorophores.

FIG. 2 illustrates fluorescence emissions of a real-time amplification assay in which a single target sequence is present in the sample being tested (out of many possible target sequences that the assay is designed to detect) and one probe (labeled with three different colors of equal fluorescence intensity) among the many different probes in the assay mixture binds to the amplified target sequence.

FIG. 3 illustrates fluorescence emissions of a real-time amplification assay in which two different target sequences are present at different concentrations in the sample being tested and two probes (each labeled with a different combination of three different colors of equal fluorescence intensity, no color being shared by both probes) bind to the amplified target sequences.

FIG. 4 illustrates fluorescence emissions of a real-time amplification assay in which two different target sequences are present at different concentrations in the sample being tested and two probes (each labeled with a different combination of three different colors of equal fluorescence intensity, one color being shared by both probes) bind to the amplified target sequences.

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FIG. 5 illustrates fluorescence emissions of a real-time amplification assay in which two different target sequences are present at the same concentration in a sample being tested, and two probes (labeled according to the first ternary color codebook shown in Fig. 6a) share two colors, leading to unambiguous interpretation of the results.

FIGS. 6a and 6b contain tables that show the effect of alternative probe coding schemes for detecting ten different possible targets in a sample, utilizing probes that are each labeled with three different colors of equal fluorescence intensity from a palette of five different fluorophores, on the results that would be obtained for every one of the 45 possible combinations of two different targets that could be present at the same concentration in a sample being tested.

FIG. 7 illustrates fluorescence emissions of a real-time amplification assay in which two different target sequences are present at the same concentration in a sample being tested, and two probes (labeled according to the first ternary color codebook shown in Fig. 6a) share one color, leading to an ambiguous result.

FIG. 8 illustrates fluorescence emissions of a real-time amplification assay in which the same sample used in the assay shown in Fig. 7 is retested using probes labeled according to the second ternary color codebook shown in Fig. 6b, providing an example of how a second assay utilizing an alternative coding scheme can resolve ambiguous results.

DETAILED DESCRIPTION

FIG. 1 is a table presenting certain combinatorial coding schemes useful in this invention. The top portion of the table presents the number of codes obtained by having each probe differently labeled with two different fluorophores from panels of 4, 5, 6, 7 or 8 fluorophores. If the panel includes eight fluorophores, there are 28 possible codes. The bottom portion of the table presents the number of codes obtained by having each probe labeled with three different fluorophores from the same panels of 5, 6, 7 or 8 fluorophores. If the panel includes eight fluorophores, there are 56 possibilities. Assays according to this invention utilize two or three (and sometimes four) differently labeled portions of each probe and a panel of fluorophores

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of sufficient size to permit at least six differently coded probes. This may be accomplished, for example, by combining the two portions of the table, that is, using both codes of two colors and codes of three colors. For example, probes may be subdivided into three portions for use with a panel of five fluorophores, providing up to ten differently coded probes, as outlined in the bottom portion of the table, and additional probes may be subdivided into two portions for use with a panel of five fluorophores, providing up to ten additional differently coded probes, as outlined in the top portion of the table. In this manner, as many as 20 differently coded probes can be combined in the same assay mixture.

Assays according to this invention utilize one probe (which may be a single-molecule probe, such as a molecular beacon probe or a TaqMan probe, or a bi-molecular probe, such as a yin-yang probe or a LightCycler probe pair) for each target. Coding is achieved, except for a code element represented by a single color, by subdividing probes into portions and labeling the portions according to a combinatorial code or combinatorial-plus-ratio code from a panel of at least four, preferably 5-8, distinguishable fluorophores. Utilizing our preferred molecular beacon probes for purposes of illustration, each probe portion is designed to have a different color signature. Certain of the probes may be wavelength-shifting probes, that is, probes in which one arm is labeled with a quencher and the other arm is labeled with a FRET pair comprising a "harvester" fluorophore to absorb energy from the stimulating light source and transfer that energy to an "emitter" fluorophore that fluoresces strongly at its own characteristic longer wavelength spectrum when the probe is in its open configuration. We utilize wavelength-shifting probes with instruments that employ a single wavelength source that is not highly absorbed by fluorophores that emit visible fluorescence at longer wavelengths, for example, Texas Red. See Tyagi, S. *et al.* (2000), Wavelength-Shifting Molecular Beacons, Nature Biotechnol. 18: 1191-1196; El-Hajj, H. *et al.* (2001), *supra*. It is also possible to utilize one fluorophore to quench another fluorophore by contact-mediated quenching. This creates two additional possibilities. First, a fluorophore that has relatively low emission in the system being used could be placed on each arm so that the fluorophores quench one another when the probe is closed but both fluoresce with the

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same color, increasing its intensity, when the probe is hybridized to its target and open. This technique can be used to add ratio coding to a combinatorial code. Second, one portion of a probe can carry two code elements (colors) by placing different fluorophores on the two arms, so that the fluorophores quench one another when the probe is closed but each fluoresces in its own characteristic emission spectrum when the probe is hybridized to its target and open. This can be used to combine two portions into one and thereby reduce the number of different portions needed for a two-color, three-color or four-color code element. It is equivalent to having separate portions. Because quenching by non-fluorescent quenchers is more efficient than quenching by fluorophores and because having two emitting fluorophores on the same molecule removes the option to balance their intensities by means of relative concentration, preferred embodiments of this invention utilize a non-fluorescent quencher on one arm of molecular beacon probes. Dabcyl is a preferred quencher, as is Black Hole Quencher No. 2.

"Codebooks" useful in this invention utilize four colors per probe, three colors per probe, two colors per probe, or combinations of four, three or two colors per probe, with or without single-color probes. Preferred codebooks utilize either only four colors per probe, only three colors per probe or only two colors per probe. The reason these are preferred is that the appearance of, for example, one color arising at a particular PCR cycle signals that an error has been made in assay preparation. If single-color probes are used, such an error is not obvious. In some embodiments of this invention, combinatorial coding may be supplemented with ratio coding to increase the number of code elements or possibilities. Preferred ratios are integral, from 5:1 to 1:5, preferably from 3:1 to 1:3. Ratio coding is most appropriate for screening assays in which it is highly likely that only one target will be found. The reason for this is that when the code is strictly combinatorial and two or more targets are present, changes in intensity levels are helpful for resolving ambiguities that may arise in analysis of the emission spectrum. Therefore, preferred assays that are not highly likely to find only one target utilize strictly combinatorial coding.

Various fluorophores can be included in panels of fluorophores. Certain fluorophores have relatively narrow emission spectra, for example, Vic, Alexa 488,

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and quantum dots (Gao *et al.* (2002), Quantum-Dot Nanocrystals for Ultrasensitive Biological Labeling and Multicolor Optical Encoding, J. Biomed. Opt. 7, 532-537), making them particularly useful for highly multiplexed assays utilizing panels of more than six fluorophores.

Oligonucleotide probes, including molecular beacon probes, are conveniently prepared by solid-phase DNA synthesis on, for example, an Applied Biosystems 394 DNA/RNA synthesizer. DNA nucleotides or RNA nucleotides may be used, including modified nucleotides such as 2'-O-methylribonucleotides, which yield strands resistant to cleavage. Our preferred method of molecular beacon synthesis starts with controlled-pore glass columns containing the quencher dabcyl for incorporation at the 3' end of the probe. These columns are available from Biosearch Technologies (Novato, California, USA). Our preferred method of incorporating fluorophores at the 5' end of the probe is to utilize thiolmodifier phosphoramidites or aminomodifier phosphoramidites so that iodoacetylated fluorophore derivatives can be coupled to the 5'-thiol groups and succinimidyl esters of fluorophores can be coupled to the 5'-amino groups. For tetrachlorofluorescein, our practice is to incorporate directly a tetrachlorofluorescein phosphoramidite. For wavelength-shifting probes a non-terminal fluorophore is required. We have used fluorescein phosphoramidites to incorporate internal fluorescein moieties for this purpose. Our molecular beacon probe synthesis includes probe purification by gel exclusion chromatography through NAP-5 Sephadex columns, followed by HPLC through a reverse-phase column such as the C-18 column marketed by Waters. Finally, probes are precipitated with ethanol and dissolved in 100 µl Tris-EDTA buffer. Molecular beacon probes should have a signal-to-noise ratio of at least 25, preferably closer to 100, keeping in mind that for a triplet code, for example, each fluorophore will be common to 30-60% of the probes.

As indicated above, the probes are subdivided into two, three or four portions for labeling and use. It is advantageous that the various portions of a probe, and where possible all portions in the assay, produce roughly the same signal intensity. Accordingly, our preferred assays and kits have portions "balanced" for that purpose. By "balanced" I mean that the fluorescence intensities of the signals from the different portions are generally within 20% of one another, most preferably within 5%. The

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fluorescence intensity produced by a particular probe portion, for example a molecular beacon probe portion, will depend on the intrinsic properties of its emitter fluorophore (and, if it is a wavelength-shifting molecular beacon probe, also its harvester fluorophore), the nature of the target, as some targets accept probes more readily than others, and quite possibly on the instrument utilized for detection. By testing a probe against its target in the assay on the instrument to be used, the amounts of the portions relative to one another can be adjusted to produce balanced signals. I prefer further that all portions of all of the combinatorially labeled probes be balanced. After the portions of each probe are balanced, the amounts of the probes relative to one another can be adjusted to produce balanced signals. It is possible to adjust signal readings by adjusting the detection instrument rather than the amounts of the portions of each probe to account for differences in the fluorophores and the instrument. For example, if a probe with three portions labeled a, b, c produces relative intensities with equal volumes of each portion of 1.0, 1.5 and 3.0, respectively, the instrument could be programmed to multiply readings from portion "a" by three, from portion "b" by two, and from portion "c" by one. Then only the relative amounts of the probes would have to be balanced. My preference, however, is to adjust the portion amounts rather than the instrument. Utilizing balanced emissions makes interpretation by a human observer significantly easier, as one can see immediately whether or not three colors, for example, indicate a particular code element.

Assays according to this invention are highly multiplexed. While not limited as to sample source, the assays are particularly applicable for use with samples from human or other animal body sites that are considered sterile, such as blood, tissue and cerebral spinal fluid. Non-sterile specimens, such as sputum or stool, are likely to have nucleic acids from a variety of sources and are more difficult to screen for that reason. Pathogens generally infect the blood stream to at least a small degree. However, nucleic acid amplification assays require minimal amounts of target sequence in the sample. For example, Kane *et al.* compared blood culturing to PCR assays utilizing "universal' bacterial primers to diagnose bacterial infection in critically ill surgical patients. Kane, T.D. *et al.* (1998), The Detection of Microbial

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DNA in Blood: a Sensitive Method for Diagnosing Bacteremia and/or Bacterial Translocation in Surgical Patients. Ann. Surg. 227: 1-9. Whereas blood cultures were positive for only 14% of the samples, the PCR assays were positive for 64%.

For embodiments employing amplification, such as PCR or NASBA, primers are required for the various possible targets. Primer design is known in the art. Primers can be tested with other primers and then with probes to check for undesired interactions. It is important that primers, and to a lesser extent probes, not interact in multiplex reactions. A type of high-specificity primer useful in this invention is disclosed in published international patent application WO 00/71562. Such primers form hairpin stems that significantly reduce undesired hybridizations. We have decreased primer interactions by utilizing such high-specificity primers, taking care to ensure that the melting temperature (T_m) of the stem is approximately 6°C above the primer annealing temperature utilized in the assay. We have constructed two pairs of universal primers that together amplify a segment of the 16s ribosomal RNA gene of many bacterial species. One pair includes 5'-TGACGACAACCATGCACC-3' (SEQ. ID NO. 1) and 5'-ATGTGGTTTAATTCGAAGCAA-3' (SEQ. ID NO. 2) to amplify target regions in *B. anthracis* and related gram-positive bacterial. The other pair includes 5'-GTGGACTTAGATACCCTGGTAGTCCAC-3' (SEQ. ID NO. 3) and 5'-GCGTTGCATCGAATTAA-3' (SEQ. ID NO. 4), which amplify target regions in several important gram-positive bacteria and gram-negative bacteria. The underlined sequences of the third identified probe are hairpin-forming sequences added to reduce undesired hybridizations by this primer.

Amplification assays with real-time detection can be performed on a variety of instruments that utilize different techniques for exciting fluorophores and detecting emissions. Excitation can be by lasers, white light coupled with filters, or light-emitting diodes. In addition, the assay instrument may utilize a synchronous scanning mode, in which narrow excitation and emission wavelengths are continuously varied, while maintaining a fixed wavelength difference between them. Lee *et al.* (1999), Seven-Color, Homogeneous Detection of Six PCR Products, Biotechniques 27, 342-349. The choice of instrumentation may affect the number of different fluorophores that can be included in a panel.

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Screening assays according to this invention, including but not limited to real-time amplification assays, may be positive for a single target, in which case the target is identifiable by its unique signature, preferably its unique color signature but possibly by its color-plus-intensity signature. On occasion two or even more than two targets may be present in a sample. Deciphering the spectrum in such a case may take advantage of the fact that, although the absolute fluorescence of a probe is dependent on target concentration, the fluorescence ratios of two probe portions hybridizing to the same target will be independent of target concentration. Taking the case of strictly combinatorial coding utilizing three-color codes wherein the intensities of the portions have been balanced, assume that two targets are present. If the targets share no color, there will result six colors. However, because equal concentrations of the two targets in the sample are highly unlikely, direct probing of the sample is likely to result in three colors of one intensity and three colors of a second intensity, thereby identifying the two targets. If the assay, on the other hand, is a real-time amplification assay, the times at which signals rise above background, for example the threshold cycle (C_T) of a PCR reaction, are likely to differ, leading to two groups of three colors, each group arising at a different time, thereby identifying the two targets.

If the codes assigned to the probes for the two targets share a color, in an assay based on directly probing the nucleic acids in a sample, the fluorescence intensity for that color will not match the fluorescence intensity for the other colors. Rather it will be a combination from two targets and will stand out from the level of the other colors, thereby identifying it as the color in common. If the assay, on the other hand, is a real-time amplification assay, three colors will come up together as signal from the more abundant target. When the second threshold for the less abundant target is reached, two more colors will come up together and the intensity curve for the common color will change slope to a higher value, thereby identifying it as the color in common.

Taking the case where ratio coding is also used, assume one color is common to two targets, but its intensity ratio for the more abundant target is 2:1 as compared to the other portions for that target. In a real-time amplification assay three colors will rise above background as the first threshold is reached, but one color (the common

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color) will have a higher slope than the other two colors. Nonetheless, when the second threshold is reached, the intensity curve of the common color will change slope to a higher value, signifying its commonality.

When two different target species are present in the sample at the same concentration, the colors encoding each probe will appear at the same time. In some instances of this situation it may not be possible to unambiguously identify each code. In such an instance a repeat assay utilizing an alternative coding scheme for the targets, should in almost all cases resolve the ambiguity.

Example 1

In this example, a real-time PCR amplification assay is carried out on DNA isolated from a normally sterile blood sample suspected of containing an infectious bacterium. The sets of primers that are present in the reaction mixture enable the amplification of a segment of the 16s ribosomal RNA gene that is present in all of the bacterial species that are suspected of possibly being present in the blood sample. A multiplicity of molecular beacon probes is present in the assay, one for each of the suspected bacterial target species. Each different molecular beacon possesses a universal quencher moiety (dabcyl) covalently linked to its 3' end. Each different species-specific molecular beacon probe is labeled combinatorially according to this invention with a unique set of three differently colored fluorophores. These fluorophores are covalently linked to the 5' end of each oligonucleotide. For every species-specific probe that is present in the assay mixture, the abundance of each of the three differently colored oligonucleotides that together make up the species-specific probe is adjusted prior to performing the assay so that the fluorescence intensity of each of the three colors of that probe will be approximately the same when measured in the assay instrument used to carry out the assay. Fig. 2 is a plot of results that will be obtained in this assay. The "x" axis represents the number of amplification cycles performed by the time that each measurement of fluorescence is made. This measurement is made automatically by the assay instrument in a manner that permits the fluorescence intensity of each of the differently colored fluorophores

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that are used to label the probes to be measured. The "y" axis represents the fluorescence intensity due a particular colored fluorophore. Measurements are taken during the annealing phase of each amplification cycle. Measurements are taken in real time during each amplification cycle throughout the course of the amplification. The fluorescence intensity of each of the differently colored fluorophores used to label the probes is plotted as a function of the number of amplification cycles completed.

In the experiment shown in Fig, 2, a signal appears that is sufficiently intense to be detected above the background fluorescence after a particular number of cycles of amplification have been carried out. The amplification cycle at which this occurs is known as the "threshold cycle", generally abbreviated as "C_T". The signal that appears after the threshold cycle consists of three different colors (a, b, and c). This signal rises in a linear fashion as more cycles of amplification are completed. The set of three colors that appear together in combination identify which bacterial species is present in the blood sample. The abundance of the species in the sample is determined from the threshold cycle at which the signal arises. The threshold cycle is inversely proportional to the logarithm of the number of target molecules in the sample

Example 2

In this example, an assay is carried out in the same manner and for the same purpose as the assay described in Example 1. The results that will be obtained in this experiment are shown in Fig. 3. Two different signals occur, one (consisting of colors a, b, and c) appears early in the reaction, and the other (consisting of colors d, e, and f) appears later in the reaction. This result indicates that two different bacterial species are present in the sample, one (identified by colors a, b, and c) is relatively abundant, and the other (identified by colors d, e, and f) is relatively rare and therefore requires more cycles of amplification before there is sufficient amplified product for the fluorescence signal due to the hybridization of the species-specific probe to be seen above the background fluorescence. The unique set of colors in

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each of the two signals unambiguously identifies each of the two bacterial species that are present in the sample.

Example 3

In this example, an assay is carried out in the same manner and for the same purpose as the assay described in Example 1. The results that will be obtained in this experiment are shown in Fig. 4. Two different signals occur, one (consisting of colors a, b, and c) appears early in the reaction, and the other (consisting of colors d and e) appears later in the reaction. In addition, the slope of the curve showing the fluorescence intensity of color "c" increases after the threshold cycle at which the later signal (consisting of colors d and e) occurs. This result indicates that two different bacterial species are present in the sample, one (identified by colors a, b, and c) is relatively abundant, and the other (identified by colors c, d, and e) is relatively rare and appears later. The increase in the slope of the curve showing the fluorescence intensity of color "c" is due the contributions of "c" fluorescence from probes binding to amplified segments of the DNA from the rare bacterial species being added the "c" fluorescence from probes binding to amplified segments of the DNA from the abundant bacterial species. Even though the three–color codes for each of the two bacterial species that are present in the sample have one color in common, the occurrence of the two species at different concentrations in the sample enables the signals from each species to be distinguished from one another. In general, when two (or even three) species are simultaneously present in a clinical sample, each will occur at a different concentration, and the coded signal due to the presence of each of the bacterial species can be distinguished from the signals due the other species in the sample.

Example 4

This example illustrates how unambiguous results can be obtained even though two different species are present in a sample at approximately the same concentration. The assay described in this example is carried out in the same manner

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and for the same purpose as the assay described in Example 1. In this experiment, the probes present in the assay mixture are designed to detect ten different bacterial species (designated species 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9). Three-color codes are used to label each of the ten probes, from a palette of five differently colored fluorophores (designated a, b, c, d, and e). For every species-specific probe that is present in the assay mixture, the abundance of each of the three differently colored oligonucleotides that together make up the species-specific probe is adjusted prior to performing the assay so that the fluorescence intensity of each of the three colors of that probe will be approximately the same when measured in the assay instrument used to carry out the assay. For this assay, the following coding scheme (which I call the "first ternary color codebook") is used:

probe for species 0	abc
probe for species 1	abd
probe for species 2	abe
probe for species 3	acd
probe for species 4	ace
probe for species 5	ade
probe for species 6	bcd
probe for species 7	bce
probe for species 8	bde
probe for species 9	cde

The results that will be obtained in the experiment carried out with probes labeled according to this codebook are shown in Fig. 5. A signal consisting of four different colors (a, b, c, and d) arises at the same threshold cycle. The slope of the curves for two of the colors (a and b) is twice as high as the slope of the curves for the other two colors (c and d). This result indicates that two different bacterial species are

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present in the sample at approximately the same concentration, and that the three-color codes of their respective probes have two colors in common. Since the slope of the curves for colors "a" and "b" is approximately twice as high as the slope of the curves for colors "c" and "e", colors "a" and "b" are components of the codes for both bacterial species that are present in the sample. The conclusion to be drawn from these results is that one of the probes that bound to amplified target sequences was encoded "abc" and the other probe that bound to amplified target sequences was encoded "abd". By comparing these results with the coding scheme in the first ternary color codebook (shown above), "abc" is seen to be the code for bacterial species "0" and "abd" is seen to be the code for bacterial species "1". Thus, the results unambiguously indicate that the sample contains species "0" and species "1" in approximately equal concentration.

Example 5

This example illustrates how ambiguous results can sometimes be obtained when two different species are present in a sample at approximately the same concentration. The assay described in this example is carried out in the same manner and for the same purpose as the assay described in Example 4. The probes used in this assay utilize the first ternary color codebook (shown in Example 4).

Fig. 6a contains a two-part table (based on the first ternary color codebook) that shows the results that should occur when two different species are present in a sample at the same concentration for each of the 45 combinations of two species that are possible. In 30 of these combinations four colors arise, two at a higher rate of fluorescence intensity increase (identified by underlined letters) and two at a lower rate of fluorescence intensity increase (identified by letters that are not underlined). Each of these 30 combinations is unique within the table, therefore providing an unambiguous result.

However, from an examination of Fig. 6a it can be seen that in 15 of the 45 possible combinations of two different species that can be present in a sample at the same concentration five colors arise, one at a higher rate of fluorescence intensity

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increase (identified by an underlined letter) and four at a lower rate of fluorescence intensity increase (identified by letters that are not underlined). These combinations, however, are not unique. For example, the code "abcde" occurs three times in the upper table: once for the combination of species "2" and "9", a second time for the combination of species "4" and "8", and a third time for the combination of species "5" and "7". An example of an experiment that yields such an ambiguous outcome is shown in Fig. 7. The results of this assay show five colors arising at the same threshold cycle (a, b, c, d, and e), one color of which (e) increases in intensity at twice the rate as the other four colors.

Example 7

The ambiguities inherent in results such as those shown in Fig. 7 can be resolved by repeating the assay with the same probes encoded by an alternative coding scheme. For example, the assay giving results shown in Fig. 8 is carried out on the same sample used to obtain the results shown in Fig. 7, except that the probes are encoded by the following alternative coding scheme (which I call the "second ternary color codebook"):

probe for species 0	cde
probe for species 1	bde
probe for species 2	bce
probe for species 3	bcd
probe for species 4	ade
probe for species 5	ace
probe for species 6	acd
probe for species 7	abe
probe for species 8	abd
probe for species 9	abc

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Fig 8 shows the results that will be obtained from an assay on the same sample used in the experiment shown in Fig. 7, except that the probes are encoded according to the second ternary color codebook. A signal consisting of four different colors (a, b, c, and e) arises at the same threshold cycle. The slope of the curves for two of the colors (b and c) is twice as high as the slope of the curves for the other two colors (a and e). Since the slope of the curves for colors "b" and "c" are approximately twice as high as the slope of the curves for colors "a" and "e", colors "b" and "c" are present in the codes for both bacterial species that are present in the sample. The conclusion drawn from these results is that one of the probes that bound to amplified target sequences is encoded "abc" and the other probe that bound to amplified target sequences is encoded "bce". By comparing these results with the coding scheme in the second ternary color codebook (shown above), "bce" is seen to be the code for bacterial species "2" and "abc" is seen to be the code for bacterial species "9". Thus, the results shown in Fig. 8 resolve the ambiguity posed by the results shown in Fig. 7, unambiguously indicating that the sample contains species "2" and species "9" in approximately equal concentration.

Fig. 6b shows the results that should occur for all 45 different combinations of two different bacterial species in a sample at approximately the same concentration when the probes are encoded according to the second ternary color codebook. A comparison of the two-part table in Fig. 6a with the two-part table in Fig. 6b shows that all 45 possible combinations of two different bacterial species can be unambiguously identified by carrying out a second assay with alternatively coded probes when it is necessary to resolve ambiguities arising from the first assay.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

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What is claimed is:

1. A highly multiplexed homogeneous *in vitro* assay to screen a sample for the presence of any of a minimum of six different nucleic acid targets comprising

a) contacting the sample with a set of fluorescent hybridization probes including at least six combinatorially coded fluorescent hybridization probes, each specific for a different nucleic acid target, wherein

i) the probes in said set are combinatorially coded with up to four different emitter fluorophores from a panel of at least four spectrally differentiable fluorophores,

ii) the probes in said set are coded by labeling portions thereof with different emitter fluorophores, and

iii) hybridization of each probe portion leads to a detectable fluorescent signal change indicative of its labeling;

b) stimulating the fluorophores present with the sample; and

c) detecting changes in emissions signals from said fluorophores without spatially segregating the nucleic acid targets from one another.

2. The assay according to claim 1 further comprising exponentially amplifying said nucleic
acid targets.

3. The assay according to claim 2 wherein changes in emission signals are detected in real time during amplification.

4. The assay according to claim 3 wherein each probe comprises two, three or four differently labeled portions.

5. The assay according to claim 1 wherein the relative amounts of the portions of each probe are adjusted relative to one another to provide balanced signal changes.

6. The assay according to claim 1 wherein said set of probes additionally includes at least one probe that is ratio coded.

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7. The assay according to claim 1 wherein said combinatorially coded probes are quenched probes whose fluorescence is restored by hybridization to their targets.

8. The assay according to claim 7 wherein said combinatorially coded probes are molecular beacon probes having a non-fluorescent quencher on one arm and an emitter fluorophore on the other arm.

9. The assay according to claim 8 wherein the fluorescence intensities of the portions of each probe are balanced relative to one another.

10. The assay according to claim 8 further comprising exponentially amplifying said nucleic acid targets in the presence of said probes and detecting increases in fluorescence from emitter fluorophores in real time.

11. The assay according to claim 10 wherein each probe comprises two, three or four differently labeled portions from a panel of four to eight differentiable fluorophores, wherein the relative amounts of the portions of each probe are adjusted relative to one another to provide balanced fluorescence emissions.

12. The assay according to claim 11 wherein the relative amounts of all portions of all probes are adjusted relative to one another to provide balanced fluorescence emissions.

13. A kit of reagents for use in a highly multiplexed *in vitro* assay to screen a sample for the presence of any of a minimum of six different nucleic acid targets comprising a set of fluorescent hybridization probes including at least six combinatorially coded fluorescent hybridization probes, each specific for a different nucleic acid target, whose hybridization to their targets can be detected in said assay by a fluorescent signal change indicative of their labeling, wherein the probes in the set are combinatorially coded with up to four different emitter fluorophores from a panel of at least four spectrally differentiable fluorophores by labeling portions of each probe with different emitter fluorophores.

14. The kit according to claim 13 further comprising primers for exponential amplification of said nucleic acid targets.

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15. The kit according to claim 14 further comprising amplification reagents for amplifying said nucleic acid targets utilizing said primers.

16. The kit according to claim 13 further comprising at least one probe that is ratio coded.

17. The kit according to claim 13 wherein the combinatorially coded probes are quenched probes whose fluorescence is restored by hybridization to their targets.

18. The kit according to claim 17 wherein said probes are molecular beacon probes having a non-fluorescent quencher on one arm and an emitter fluorophore on the other arm.

19. The kit according to claim 18 wherein the relative amounts of the portions of each probe are adjusted relative to one another to provide balanced fluorescence emissions.

20. The kit according to claim 19 wherein the relative amounts of all portions of all probes are adjusted relative to one another to provide balanced fluorescence emissions.

21. The kit according to claim 18 further comprising primers for exponential amplification of said nucleic acid targets.

22. The kit according to claim 21 further comprising amplification reagents for amplifying said nucleic acid targets utilizing said primers.

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TWO COLORS PER PROBE:
PANEL OF 4(a, b, c, d), 5(+e), 6(+f), 7(+g) or 8(+h) FLUOROPHORES

ab					}= 6	}	}	}	}					
ac	bc				}	}	}	}	}					
ad	bd	cd			}	} = 10	}	}	}					
						}	}	}	}					
ae	be	ce	de			}	} =15	}	}					
							}	}	}					
af	bf	cf	df	ef			}	} =21	}					
								}	}					
ag	bg	cg	dg	eg	fg			}	}= 28					
									}					
ah	bh	ch	dh	eh	fh	gh			}					

THREE COLORS PER PROBE:
PANEL OF 5(a, b, c, d, e), 6(+f), 7(+g) or 8(+h) FLUOROPHORES

abc					} =10	}	}	}	}											
abd					}	}	}	}	}											
abe					}	}	}	}	}											
acf	bcd				}	}	}	}	}											
ace	bce				}	}	}	}	}											
ade	bde	cde			}	}	}	}	}											
						}= 20	}	}	}											
abf						}	}	}	}											
acf	bcf					}	}	}	}											
adf	bdf	cdf				}	}	}	}											
aef	bef	cef	def			}	}	}	}											
							}= 35	}	}											
abg							}	}	}											
acg	bcg						}	}	}											
adg	bdg	cdg					}	}	}											
aeg	beg	ceg	deg				}	}	}											
afg	bfg	cfg	dfg	efg			}	}	}											
									}											
abh									}											
ach	bch								}= 56											
adh	bdh	cdh							}											
aeh	beh	ceh	deh						}											
afh	bfh	cfh	dfh	efh					}											
agh	bgh	cgh	dgh	egh	fgh				}											

FIGURE 1

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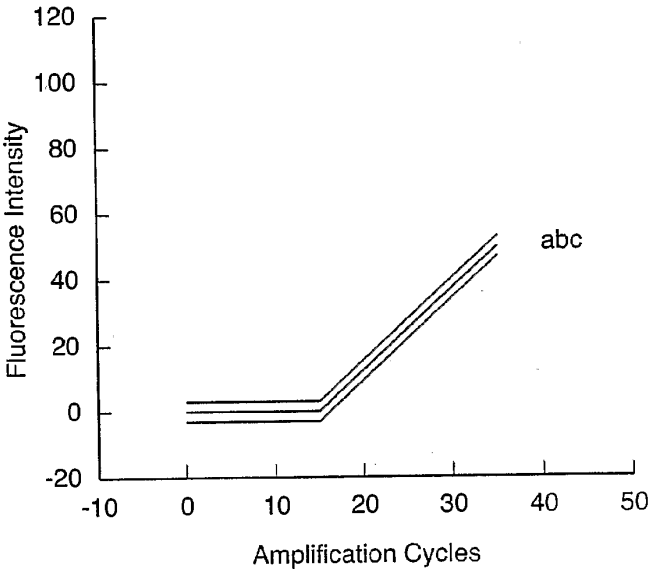


FIGURE 2

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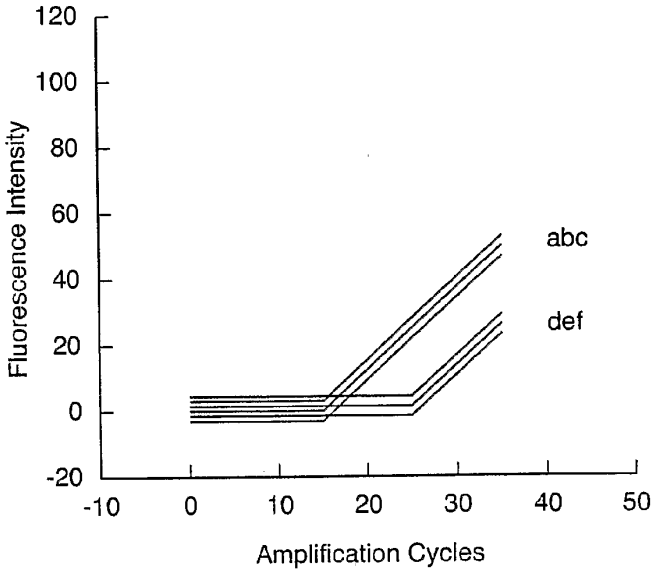


FIGURE 3

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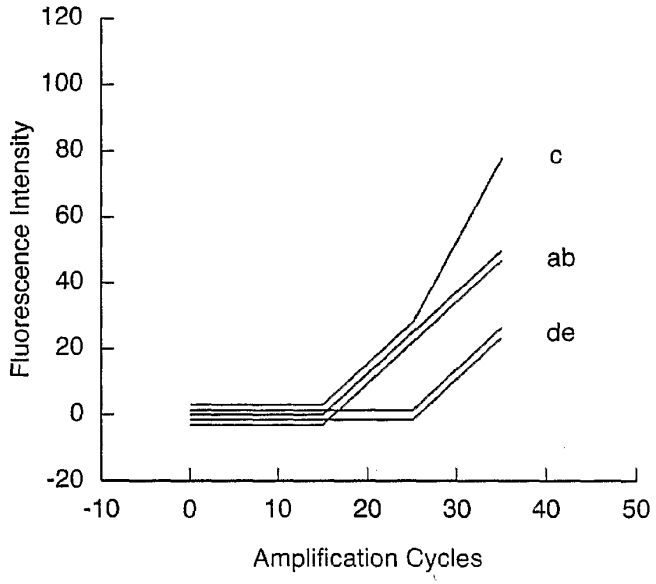


FIGURE 4

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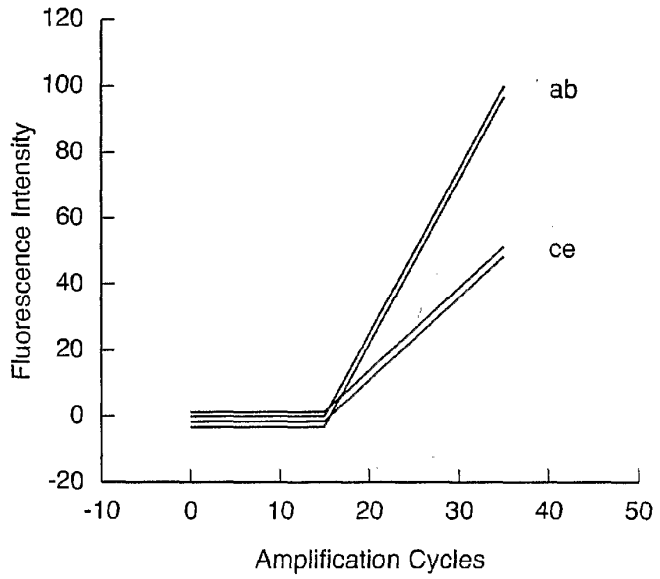


FIGURE 5

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FIRST TERNARY COLOR CODEBOOK

	0(abc)	1(abd)	2(abe)	3(acd)	4(ace)
0(abc)		0+1(abcd)	0+2(abce)	0+3(abcd)	0+4(abce)
1(abd)	0+1(abcd)		1+2(abde)	1+3(abcd)	1+4(abcde)
2(abe)	0+2(abce)	1+2(abde)		2+3(abcde)	2+4(abce)
3(acd)	0+3(abcd)	1+3(abcd)	2+3(abcde)		3+4(acde)
4(ace)	0+4(abce)	1+4(abcde)	2+4(abce)	3+4(acde)	
5(ade)	0+5(abcde)	1+5(abde)	2+5(abde)	3+5(acde)	4+5(acde)
6(bcd)	0+6(abcd)	1+6(abcd)	2+6(abcde)	3+6(abcd)	4+6(abcde)
7(bce)	0+7(abce)	1+7(abcde)	2+7(abce)	3+7(abcde)	4+7(abce)
8(bde)	0+8(abcde)	1+8(abde)	2+8(abde)	3+8(abcde)	4+8(abcde)
9(cde)	0+9(abcde)	1+9(abcde)	2+9(abcde)	3+9(acde)	4+9(acde)

	5(ade)	6(bcd)	7(bce)	8(bde)	9(cde)
0(abc)	0+5(abcde)	0+6(abcd)	0+7(abce)	0+8(abcde)	0+9(abcde)
1(abd)	1+5(abde)	1+6(abcd)	1+7(abcde)	1+8(abde)	1+9(abcde)
2(abe)	2+5(abde)	2+6(abcde)	2+7(abce)	2+8(abde)	2+9(abcde)
3(acd)	3+5(acde)	3+6(abcd)	3+7(abcde)	3+8(abcde)	3+9(acde)
4(ace)	4+5(acde)	4+6(abcde)	4+7(abce)	4+8(abcde)	4+9(acde)
5(ade)		5+6(abcde)	5+7(abcde)	5+8(abde)	5+9(acde)
6(bcd)	5+6(abcde)		6+7(bcde)	6+8(bcde)	6+9(bcde)
7(bce)	5+7(abcde)	6+7(bcde)		7+8(bcde)	7+9(bcde)
8(bde)	5+8(abde)	6+8(bcde)	7+8(bcde)		8+9(bcde)
9(cde)	5+9(acde)	6+9(bcde)	7+9(bcde)	8+9(bcde)	

FIGURE 6a

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SECOND TERNARY COLOR CODEBOOK

	9(abc)	8(abd)	7(abe)	6(acd)	5(ace)
9(abc)		8+9(abcd)	7+9(abce)	6+9(abcd)	5+9(abce)
8(abd)	8+9(abcd)		7+8(abde)	6+8(abcd)	5+8(abcde)
7(abe)	7+9(abce)	7+8(abde)		6+7(abcde)	5+7(abce)
6(acd)	6+9(abcd)	6+8(abcd)	6+7(abcde)		5+6(acde)
5(ace)	5+9(abce)	5+8(abcde)	5+7(abce)	5+6(acde)	
4(ade)	4+9(abcde)	4+8(abde)	4+7(abde)	4+6(acde)	4+5(acde)
3(bcd)	3+9(abcd)	3+8(abcd)	3+7(abcde)	3+6(abcd)	3+5(abcde)
2(bce)	2+9(abce)	2+8(abcde)	2+7(abce)	2+6(abcde)	2+5(abce)
1(bde)	1+9(abcde)	1+8(abde)	1+7(abde)	1+6(abcde)	1+5(abcde)
0(cde)	0+9(abcde)	0+8(abcde)	0+7(abcde)	0+6(acde)	0+5(acde)

	4(ade)	3(bcd)	2(bce)	1(bde)	0(cde)
9(abc)	4+9(abcde)	3+9(abcd)	2+9(abce)	1+9(abcde)	0+9(abcde)
8(abd)	4+8(abde)	3+8(abcd)	2+8(abcde)	1+8(abde)	0+8(abcde)
7(abe)	4+7(abde)	3+7(abcde)	2+7(abce)	1+7(abde)	0+7(abcde)
6(acd)	4+6(acde)	3+6(abcd)	2+6(abcde)	1+5(abcde)	0+6(acde)
5(ace)	4+5(acde)	3+5(abcde)	2+5(abce)	1+5(abcde)	0+5(acde)
4(ade)		3+4(abcde)	2+4(abcde)	1+4(abde)	0+4(acde)
3(bcd)	3+4(abcde)		2+3(bcde)	1+3(bcde)	0+3(bcde)
2(bce)	2+4(abcde)	2+3(bcde)		1+2(bcde)	0+2(bcde)
1(bde)	1+4(abde)	1+3(bcde)	1+2(bcde)		0+1(bcde)
0(cde)	0+4(acde)	0+3(bcde)	0+2(bcde)	0+1(bcde)	

FIGURE 6b

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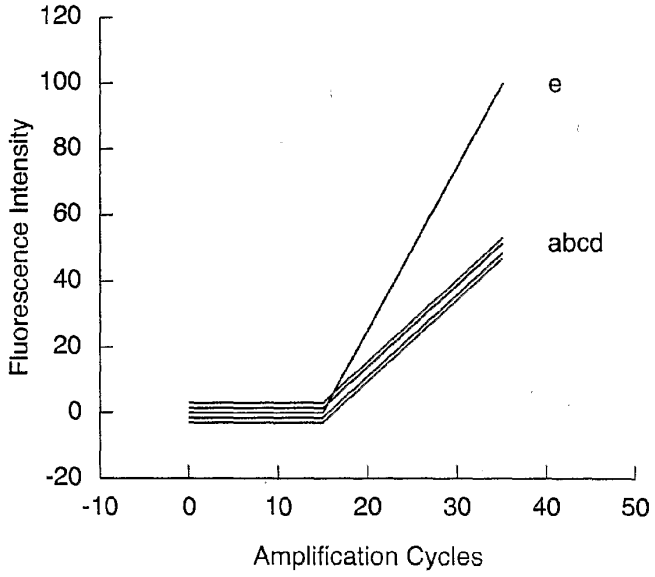


FIGURE 7

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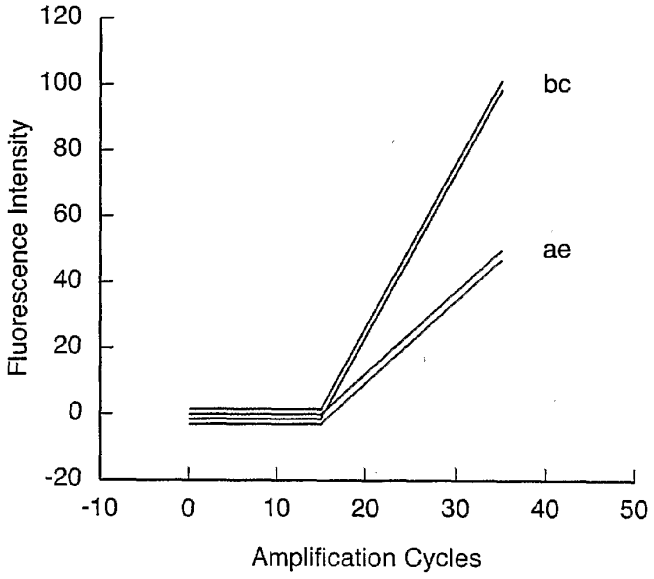


FIGURE 8

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Espacenet

CN102559868A Method for qualitative and quantitative detection of multiple target nucleotide sequences with single tube

Applicants: UNIV XIAMEN

Inventors: QINGGE LI,YIQUN LIAO

Classifications:
IPC ***C12Q1/68;***

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Publication: CN102559868A·2012-07-11

Published as: CN102559868A;**CN102559868B**

Method for qualitative and quantitative detection of multiple target nucleotide sequences with single tube

Abstract

The invention discloses a method for qualitative and quantitative detection of multiple target nucleotide sequences with a single tube, which comprises the following steps of: 1) selecting a relative conservative area in a target gene sequence to design an universal amplimer; 2) selecting an area with high diversity in primer amplified product to design specific probes capable of identifying various target nucleotide sequences, and at the same time designing multiple detecting passages, and designing to divide the probes into multiple groups, wherein, the probe of each group is provided with a same fluorescent group as a detecting passage, and melting point (Tm) value of every probe is artificially controlled by adjusting length and/or basic components of the probe during design process of the probe so as to obtain appropriate differentials between the Tm value of the detecting probes specific for various target sequences in the same detecting passage; 3) analyzing and identifying the target sequences through melting point curve. Every fluorescent passage within the single tube disclosed by the invention can perform the qualitative detection of multiple target nuclear sequences; as well as can determine copy number of each target nucleotide sequence; and can judge ratios between the target sequences under the condition of coexistence of the multiple target sequences.

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DESCRIPTION CN102559868A

10 A method for qualitatively and quantitatively detecting multiple target nucleic acid sequences in a single tube

[0001]

14 Technical Field

[0002]

18 The present invention relates to a medium-throughput real-time fluorescence quantitative PCR detection method which combines the real-time fluorescence quantitative PCR technology with the probe melting curve technology, and its main feature is that it can simultaneously perform quantitative detection on the basis of identifying multiple target objects.

22 The invention belongs to the field of biotechnology.

[0003]

26 Background Art

[0004]

30 Since nucleic acid is the basic genetic material of organisms, the identification and quantification of nucleic acid sequences is a very important technology in molecular biology research.

32 Currently, there are mainly the following types of technologies for identifying or quantifying nucleic acid sequences: (1) nucleic acid sequencing technology; (2) solid phase hybridization technology; (3) conventional PCR product analysis technology; (4) real-time fluorescence PCR technology.

[0005]

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38 Nucleic acid sequencing technology is considered to be the gold standard technology for nucleic acid sequence identification.

38 Due to its high accuracy and completeness of sequence information in the case of a single detection object, it is the main technology used in basic research to explore unknown nucleic acid sequences and identify the reliability of other detection methods.

43 However, the detection cost of nucleic acid sequencing technology is relatively high. In 1998, Mostafa Ronaghi et al. reported a new pyrophosphate sequencing technology in Science that reduced the sequencing cost, but the detection length of the nucleic acid sequence also decreased. Sequencing technology also has the following disadvantages: the sample to be tested must have a certain purity and concentration; quantitative detection cannot be performed; when two or more similar sequences exist in the nucleic acid sample at the same time, overlapping peaks will occur, thus affecting the accuracy of the results.

[0006]

52 Solid phase hybridization technology is currently a commonly used technology for nucleic acid sequence identification. This type of technology captures specific nucleic acid sequences on a solid phase carrier through the specific binding ability of complementary nucleic acid sequences, thereby achieving specific identification of nucleic acid sequences.

56 This technology has the advantages of moderate cost, mature operating procedures and resistance to impurities in clinical specimens, so it is currently adopted by many companies, such as Roche's Linear array series kits, Innogenetics' LIPA series kits and Qiagen's HC-II kits. The detection throughput of solid phase hybridization technology can be greatly improved by improving the type of solid phase carrier: for example, gene chip technology and Luminex's flow cytometry liquid phase chip technology, but the cost of these new solid phase carriers is relatively high. Although solid-phase hybridization technology is mature and has a high detection throughput, the operation steps are relatively cumbersome and quantitative detection cannot be performed. Since the detection sensitivity of simple solid phase hybridization technology is not high, it is often necessary to combine it with PCR amplification technology in practical applications.

[0007]

68 Conventional PCR product analysis technology is a nucleic acid sequence recognition technology widely used in scientific research.

70 PCR technology can quickly replicate a large number of DNA fragments with the same sequence and therefore has a high detection sensitivity. By rationally designing specific primers, only the specific nucleic acid sequence of the sample can be amplified. By judging the length of the PCR product through electrophoresis technology, it can be determined what kind of nucleic acid sequence is contained in the sample. Mass spectrometry has a much higher resolution than electrophoresis, and therefore can also analyze base mutations in PCR products of the same length (MS Bray article). However, ordinary PCR technology requires post-PCR processing to obtain information about PCR products, which can easily cause contamination of PCR products. The emergence of real-time fluorescence PCR technology makes up for this shortcoming.

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[0008]

Real-time fluorescence PCR technology is currently an important method for nucleic acid sequence detection. This technology can not only identify specific nucleic acid sequences but also determine the concentration of specific nucleic acid sequences.

Because of its advantages such as high sensitivity, good specificity, short time consumption, no need for PCR post-processing and easy operation, this technology is widely used in scientific research. However, due to the limitation of the number of detection channels of the real-time fluorescence PCR instrument, the detection throughput of traditional real-time fluorescence PCR technology is not high. Currently, the mainstream real-time fluorescence PCR on the market generally has only 2-6 detection channels, which makes it impossible for this technology to simultaneously detect more target nucleic acid sequences in a single tube. Therefore, using various methods to improve the detection throughput of real-time fluorescence PCR technology has become a research hotspot for scientific researchers.

[0009]

In summary, although there are a variety of nucleic acid sequence identification and quantitative detection technologies at home and abroad, they all have certain limitations. Therefore, it is necessary to develop a nucleic acid sequence detection technology that can be both quantitative and qualitative, high-throughput, simple to operate and low-cost.

[0010]

Summary of the invention

[0011]

The main purpose of the invention is to design a novel real-time fluorescent PCR probe melting curve DNA typing technology.

[0012]

The technical solution of the present invention is as follows:

[0013]

A method for qualitatively and quantitatively detecting multiple target nucleic acid sequences in a single tube comprises the following steps:

[0014]

(1) Design universal amplification primers from relatively conserved regions in the target gene sequence;

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[0015]

125 (2) selecting regions with large differences in the amplified fragments to design specific probes that can identify various target nucleic acid sequences. During the probe design process, the melting point (Tm) value of each probe is artificially controlled by adjusting the length of the probe and/or the composition of the probe bases, so that the Tm values of the detection probes for different target sequences in the same detection channel are spaced by appropriate differences; and multiple detection channels are designed at the same time;

[0016]

133 (3) The target sequence was determined by melting point curve analysis.

[0017]

137 More preferably, when detecting the target nucleic acid sequence, a detection channel is added to detect the melting curve signal of the internal standard of the system to ensure the effectiveness of the system.

[0018]

142 In the present invention, the detection channel refers to the ability of the instrument to resolve different fluorescent groups.

144 The number of fluorescent probe types that can be used is determined by the number of fluorescence signal acquisition channels of the real-time fluorescence PCR instrument.

146 Existing real-time fluorescence PCR instruments can usually detect 2-6 different fluorescent dyes simultaneously. Therefore, depending on the number of target sequences, the number of detection channels can be designed to be 2-6.

[0019]

152 In the present invention, the types of fluorescent groups include but are not limited to: ALEX-350, FAM, VIC, TET, CAL Fluor Gold 540, JOE, HEX, CAL Flour Orange 560, TAMRA, Cal Fluor Red 590, ROX, CAL Fluor Red 610, TEXAS RED, CAL Flour Red 635, Quasar 670, CY3, CY5, CY5.5 or Quasar 705; the quenching groups used for the above fluorescent groups include but are not limited to DABCYL, BHQ, ECLIPSE or TAMRA.

[0020]

160 In the present invention, the interval difference of Tm values of adjacent probes in the same detection channel may be the same or different, and the interval difference of Tm values of probes in different detection channels may be the same or different.

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[0021]

165 In the present invention, the interval range of the Tm value is determined by the melting curve resolution capability of the real-time fluorescence PCR instrument.

168 Generally, the Tm values of probes in the same detection channel may differ by 1° C to 15° C.

[0022]

172 More preferably, the Tm values of the probes in the same detection channel have a difference of 3° C-8° C.

[0023]

177 In the present invention, preferably, the probe length is 18 bp to 50 bp.

[0024]

181 In the present invention, preferably, the probe has a G+C base content of 30%-60%.

[0025]

185 The technical principle of the present invention is as follows: As shown in FIG1, the present invention first selects a relatively conservative region in the target gene sequence to design universal amplification primers.

187 Then, a region with a large difference is selected from the primer amplification product to design a specific probe capable of identifying various target nucleic acid sequences. During the probe design process, the present invention artificially controls the melting point temperature (Tm value) of each probe by adjusting the length of the probe (18 bp to 50 bp) and the content of the G+C base of the probe (30% to 60%), so that the Tm values of the detection probes for different target sequences in the same detection channel are spaced at appropriate differences, and the interval range of the Tm value is determined by the melting curve resolution capability of the real-time fluorescence PCR instrument.

194 When detecting the target nucleic acid sequence, the present invention particularly adds a detection channel for detecting the melting curve signal of the internal standard of the system to ensure the effectiveness of the system.

197 Finally, the melting curve signal is optimized by adjusting the PCR amplification system and various conditions in the PCR amplification program. Another purpose of the present invention is to provide the concept of using probes of different lengths (or base compositions) to cause differences in the Tm values of the melting curve to achieve multiple detections in a single fluorescent channel.

[0026]

204 The present invention adjusts the Tm value of the probe melting curve by changing the probe length, so that each Tm value corresponds to a target DNA sequence, thereby realizing qualitative detection of the target sequence; by customizing the real-time fluorescence PCR amplification Ct value working curve of each target

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sequence, qualitative detection of each target sequence is realized; and by comparing the peak heights of the melting curves generated by different target sequences and probes, the ratio judgment between multiple target sequences is realized.

The present invention combines qualitative detection with quantitative detection by combining real-time fluorescence quantitative PCR technology with probe melting curve technology.

The present invention determines the proportional relationship between multiple target DNA sequences based on the relative comparison of probe melting curve peaks.

[0027]

The technology of the present invention has the following advantages: (1) Due to the difference in Tm values between detection probes labeled with the same fluorescent dye, the detection throughput is increased several times compared with the traditional real-time fluorescent PCR technology, while ensuring the same detection sensitivity; (2) Since the Tm value and Ct value are used for qualitative and quantitative detection in a single tube respectively, the technology has a very high detection accuracy; (3) Since a single tube can detect multiple types of target sequences in parallel at the same time, the operation amount and detection cost of the detection technology are greatly reduced.

(4) This technology can obtain triple results of qualitative detection, quantitative detection and mixed infection detection through a single tube and one reaction, and the required detection time is basically the same as that of traditional real-time fluorescence PCR technology.

(5) Compared with traditional fluorescent PCR technology, this technology has better tolerance for inter-type polymorphic sites.

[0028]

BRIEF DESCRIPTION OF THE DRAWINGS

[0029]

Fig. 1 is a technical principle diagram of the present invention;

[0030]

FIG2 is a test result of Example 1 of the present invention;

[0031]

FIG3 is a test result of the second embodiment of the present invention;

[0032]

FIG4 is a test result of Example 3 of the present invention;

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[0033]

255 FIG. 5 is the detection result of the fourth embodiment of the present invention.

[0034]

259 DETAILED DESCRIPTION

[0035]

263 In order to further illustrate the present invention, we will describe it in the following embodiments.

264 The following examples all used ABI7500 real-time fluorescence PCR instrument to complete the detection of amplification curves and melting curves.

[0036]

269 Detection system: 1*PCR buffer, 2.5mM magnesium ions, 1U Taq DNA polymerase, 200μM dNTP, 1pmol upstream primer, 20pmol downstream primer, 5pmol detection probe.

[0037]

274 Detection procedure: pre-denaturation at 95° C for 3 minutes; denaturation at 95° C for 10 seconds, annealing at 48° C for 15 seconds, annealing at 72° C for 20 seconds, and fluorescence detection during annealing at 48° C, repeat 45 cycles; melting curve analysis at 40° C-85° C, and fluorescence signal detection every 1° C.

[0038]

281 Embodiment 1:

[0039]

285 The single-tube three-color system qualitatively detects positive plasmid standards and internal standard genes of 10 HPV types.

[0040]

290 The experimental process is as follows:

[0041]

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294 (1) Construction of positive plasmids: DNA sequences were synthesized based on the DNA sequences of 10 HPV genotypes, including HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-58, HPV-59, HPV-68, HPV-73, and HPV-82, in the amplification region of the GP5+/GP6+ primers (sequences are as follows: GP5+: 5' -TTTGTTACTGTGGTAGATACTAC-3', GP6+: 5' -GAAAAATAAACTGTAAATCATATTC-3') and partial DNA sequences of the internal standard human β-globin gene, and then inserted into the SmaI cloning site of the pUC57 plasmid vector.

294 The detection probes for 10 HPV types and their corresponding Tm values and temperatures are shown in Table 1.

[0042]

305 (2) PCR amplification and melting curve analysis: The system for PCR amplification and melting curve analysis was as described above. The amount of sample added to each reaction system was 1*10⁶copies/μl of HPV standard plasmids of different types and 5 μl of internal standard plasmid. The detection procedure was as described above.

[0043]

312 (3) Result interpretation: As shown in Figure 1, the HPV type in the sample can be interpreted based on the characteristic Tm value of each HPV type in a specific detection channel; if there is only an internal standard detection signal but no HPV type characteristic peak, it means that the sample does not carry these 10 HPV types; if there is neither an internal standard detection signal nor an HPV type detection signal, it means that PCR amplification has failed.

[0044]

320 As shown in Figure 2, the detection system can detect specific melting curve detection signals for all 10 HPV types, and there is no cross-detection between different HPV types. In the negative control, there is only a melting curve detection signal of the internal standard β-globin gene.

[0045]

326 Table 1: Detection probes for 10 HPV types and their corresponding Tm values

[0048]

330 Embodiment 2:

[0049]

334 Single-tube three-color system for quantitative detection of positive plasmid standards of 10 HPV types.

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[0050]

339 The design of primers and probes was the same as in Example 1.

[0051]

343 (1) PCR amplification detection: The system and procedure of PCR amplification were as described above. The amount of sample added to each reaction system was 5 μl of standard plasmids of various HPV types with a concentration of 1*10²—1*10⁶copies/μl.

[0052]

349 (2) Working curve preparation: As shown in FIG3, quantitative detection working curves corresponding to various HPV types were prepared based on the Ct values of PCR amplification after gradient dilution of standard plasmids of various HPV types.

[0053]

355 (3) Result interpretation: Based on the HPV type determined in the results of Example 1, the viral load in the sample is further determined by the corresponding position of the sample PCR amplification Ct value on the working curve.

[0054]

361 As shown in Figure 3, the detection system can perform quantitative analysis while typing 10 HPV types.

362 The corresponding relationships between the amplification curve, working curve and HPV type are: (A) HPV-18, (B) HPV-35, (C) HPV-16, (D) HPV-33, (E) HPV-31, (F) HPV-73, (G) HPV-59, (H) HPV-82, (I) HPV-58, and (J) HPV-68.

[0055]

368 Real-time example three:

[0056]

372 The design of primers and probes was the same as in Example 1.

[0057]

376 Mixed infection detection of HPV-16 and HPV-18 in different proportions (0%, 1%, 3%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 97%, 99%, 100%).

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[0058]

382 The experimental process is as follows:

[0059]

386 (1) PCR amplification detection: The system and procedure of PCR amplification and melting curve analysis were as described above. The amount of sample added to each reaction system was 5 μl of 1*10⁶copies/μl HPV-16 and HPV-18 mixed standard plasmid.

[0060]

392 (2) Result interpretation: As shown in FIG4, the virus type is determined by the Tm value range, and the viral load ratio of multiple infections is determined by the relative height of the peak value.

[0061]

397 As shown in FIG4, the lower limit of the detection capability of the detection system for mixed infection is 3%.

[0062]

402 Example 4: Qualitative detection of 9 common thrips.

[0063]

406 The experimental process is as follows:

[0064]

410 (1) Construction of positive plasmids: Based on the target DNA sequence in the COI (cytochrome oxidase) region of the mitochondrial genes of nine species of thrips, including flower thrips, grass thrips, western flower thrips, tobacco thrips, palm thrips, yellow thrips, yellow-breasted thrips, onion thrips, and common giant thrips in the NCBI gene bank (http://www.ncbi.nlm.nih.gov), the PF1/PR1 (PF1: 5' -ATTAGGAGCHCCHGAYATAGCATT-3' PR1: 5' -CAGGCAAGATTAAAATATAAACTTGG-3') primer amplification region and the partial DNA sequence of the internal standard 16S rDNA gene were synthesized and inserted into the SmaI cloning site of the pUC57 plasmid vector.

[0065]

420 (2) PCR amplification and melting curve analysis: The system for PCR amplification and melting curve analysis was as described above. The amount of sample added to each reaction system was

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1*10⁶ copies/μl of different types of HPV standard plasmids and 5 μl of internal standard plasmid. The detection procedure was as described in the content of the invention.

[0066]

429 (3) Result interpretation: As shown in Figure 1, the type of thrips to which the sample belongs can be judged based on the characteristic Tm value of each thrips species in a specific detection channel; if there is only an internal standard detection signal but no characteristic peak of the thrips species, it means that the sample does not belong to these 9 types of thrips; if there is neither an internal standard detection signal nor a thrips species detection signal, it means that PCR amplification has failed.

[0067]

437 Note: 1 = flower thrips, 2 = grass thrips, 3 = western flower thrips, 4 = tobacco thrips, 5 = palm thrips, 6 = yellow thrips, 7 = yellow-breasted thrips, 8 = onion thrips, 9 = common macrothrips, 10 = internal standard

[0068]

443 As shown in FIG5, the detection system can detect specific melting curve detection signals for all nine species of thrips, and there is no cross-detection phenomenon between different thrips species. In the negative control, there is only the melting curve detection signal of the internal standard.

[0069]

449 The detection probes and corresponding Tm values of 9 species of thrips are shown in Table 2.

[0070]

453 Table 2: Detection probes for 9 species of thrips and their corresponding Tm values and temperatures

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(54)发明名称

一种单管定性并定量检测多个目标核酸序列的方法

(57)摘要

本发明公开了一种单管定性并定量检测多个目标核酸序列的方法，其包括如下步骤：(1)在目标对象基因序列中选取相对保守的区域设计通用扩增引物；(2)在引物扩增产物中选取差异度较大的区域设计能够识别各种目标核酸序列的特异探针，同时设计多个检测通道，将探针设计为多组，每组的探针配备相同的荧光基团作为一个检测通道，在探针设计过程中通过调整探针的长度和/或探针的碱基组成对每条探针的熔点(Tm)值进行人为控制，使得同一检测通道内针对不同目标序列的检测探针 Tm 值都间隔合适的差值；(3)通过熔点曲线分析分辨靶序列。本发明单个反应管内的每个荧光通道均可定性检测多个目标核酸序列；同时能够确定各个目标核酸序列的拷贝数；在多个目标序列共存的情况下能够判断目标序列间的比例。

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1. 一种单管定性并定量检测多个目标核酸序列的方法，包括如下步骤：

(1) 在目标对象基因序列中选取相对保守的区域设计通用扩增引物；

(2) 在引物扩增产物中选取差异度较大的区域设计能够识别各种目标核酸序列的特异探针，同时设计多个检测通道，将探针设计为多组，每组的探针配备相同的荧光基团作为一个检测通道，在探针设计过程中通过调整探针的长度和 / 或探针的碱基组成对每条探针的熔点值进行人为控制，使得同一检测通道内针对不同目标序列的检测探针 Tm 值都间隔合适的差值；

(3) 通过熔点曲线分析分辨靶序列。

2. 如权利要求 1 所述的一种单管定性并定量检测多个目标核酸序列的方法，其特征在于：在检测目标核酸序列时，还增加一个检测通道，用于检测体系内标的熔解曲线信号以保证体系的有效性。

3. 如权利要求 1 所述的一种单管定性并定量检测多个目标核酸序列的方法，其特征在于：所述的探针为荧光标记探针。

4. 如权利要求 1 或 2 所述的一种单管定性并定量检测多个目标核酸序列的方法，其特征在于：所述的检测通道为 2-6 个。

5. 如权利要求 1 所述的一种单管定性并定量检测多个目标核酸序列的方法，其特征在于：同一检测通道相邻探针的 Tm 值的间隔差值可以是相同的或是不同的，不同检测通道探针的 Tm 值间隔差值可以是相同的或是不同的。

6. 如权利要求 1 所述的一种单管定性并定量检测多个目标核酸序列的方法，其特征在于：同一检测通道探针的 Tm 值的间隔差值为 1℃ -15℃。

7. 如权利要求 1 所述的一种单管定性并定量检测多个目标核酸序列的方法，其特征在于：所述的 Tm 值的间隔差值为 2℃ -8℃。

8. 如权利要求 1 所述的一种单管定性并定量检测多个目标核酸序列的方法，其特征在于：所述探针长度为 18bp 到 50bp。

9. 如权利要求 1 所述的一种单管定性并定量检测多个目标核酸序列的方法，其特征在于：探针 G+C 碱基的含量 30％ -60％。

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一种单管定性并定量检测多个目标核酸序列的方法

技术领域

[0001] 本发明涉及一种将实时荧光定量 PCR 技术与探针熔解曲线技术结合使用的中通量实时荧光定量 PCR 检测方法，其主要特征为在能够在识别多个目标对象的基础上同时进行定量检测。本发明属于生物技术领域。

背景技术

[0002] 由于核酸是生物的基本遗传物质，因此核酸序列的识别与定量是分子生物学研究中一项非常重要的技术。目前核酸序列的识别或定量技术主要有以下几类：(1) 核酸测序技术；(2) 固相杂交技术；(3) 普通 PCR 产物分析技术；(4) 实时荧光 PCR 技术。

[0003] 核酸测序技术被认为是目前核酸序列识别的金标准技术。由于在单一检测对象的情况下具有高度的准确性和序列信息的完整性，因此在基础研究中被用于探索未知核酸序列和鉴定其他检测方法可靠性的主要技术。不过核酸测序技术的检测成本较高，对此 1998 年 Mostafa Ronaghi 等在《Science》上报导了一种新型的焦磷酸测序技术降低了测序成本不过对于核酸序列的检测长度也随之下降。测序技术同时也存在以下几个缺点：要求待检标本必须具备一定的纯度和浓度；无法进行定量检测；核酸样品中同时存在两个或多个近似序列时会出现套峰从而影响结果准确性。

[0004] 固相杂交技术是目前核酸序列识别一种常用的技术，该类技术通过互补核酸序列特异性结合能力将特定核酸序列捕获在固相载体上，从而实现核酸序列的特异识别。该技术具有成本适中、操作流程成熟并且耐临床标本中的杂质等优点，所以为目前许多公司所采用，例如 Roche 公司的 Linear array 系列试剂盒，Innogenetics 公司的 LIPA 系列试剂盒以及 Qiagen 公司的 HC-II 试剂盒。通过改进固相载体的类型可以大幅度提高固相杂交技术的检测通量：例如基因芯片技术以及 Luminex 公司的流式液相芯片技术，不过这些新型的固相载体所耗费的成本都比较高。固相杂交技术虽然技术成熟并且检测通量高，但是操作步骤较为繁琐并且无法进行定量检测。由于单纯的固相杂交技术的检测灵敏度并不高，因此在实际应用中常常需要和 PCR 扩增技术进行联用。

[0005] 普通 PCR 产物分析技术是目前科学研究中广泛应用的核酸序列识别技术。PCR 技术能够快速复制出大量相同序列 DNA 片段因此具备有很高的检测灵敏度。通过合理设计特异引物可以只对样品特定的核酸序列进行扩增，通过电泳技术对 PCR 产物的长度进行判断，即可判断样品中是带有何种核酸序列。质谱技术拥有比电泳高的多的分辨率，因此还可分析相同长度的 PCR 产物中的碱基突变情况 (MS Bray 文章)。不过普通 PCR 技术需要进行 PCR 后处理才能获得 PCR 产物的信息，因此容易造成 PCR 产物的污染现象。而实时荧光 PCR 技术的出现弥补了这一缺陷。

[0006] 实时荧光 PCR 技术是目前核酸序列检测的一个重要方法，该技术不仅可以识别特定核酸序列而且可以确定特定核酸序列的浓度。由于该技术具有灵敏度高、特异性好、耗时短、无需 PCR 后处理以及操作简便等优点，在科研工作中被广泛使用。不过由于实时荧光 PCR 仪的检测通道数量的限制，传统的实时荧光 PCR 技术的检测通量并不高。目前市面上

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主流的实时荧光 PCR 的检测通道一般只有 2-6 个，导致该技术无法单管同步检测较多的目标核酸序列。因此采用各种方法提高实时荧光 PCR 技术检测通量成为科研工作者的研究热点。

[0007] 综上所述，虽然目前国内外已有多种核酸序列识别与定量的检测技术，但都具有一定的局限性，所以有必要开发一种既能定量又能定性、高通量、操作简单而且低成本的核酸序列检测技术。

发明内容

[0008] 本发明的主要目的是设计一种新型的实时荧光 PCR 探针熔解曲线 DNA 分型技术。

[0009] 本发明的技术方案如下：

[0010] 一种单管定性并定量检测多个目标核酸序列的方法，包括如下步骤：

[0011] (1) 在目标对象基因序列中选取相对保守的区域设计通用扩增引物；

[0012] (2) 在扩增片段中选取差异度较大的区域设计能够识别各种目标核酸序列的特异探针，在探针设计过程中通过调整探针的长度和 / 或探针碱基的组成对每条探针的熔点 (Tm) 值进行人为控制，使得同一检测通道内针对不同目标序列的检测探针 Tm 值都间隔合适的差值；同时设计多个检测通道；

[0013] (3) 通过熔点曲线分析分辨靶序列。

[0014] 更佳地，在检测目标核酸序列时，还增加一个检测通道用于检测体系内标的熔解曲线信号以保证体系的有效性。

[0015] 在本发明中，所述的检测通道即仪器对不同的荧光基团的分辨能力。可使用的检测荧光探针类型的数量由实时荧光 PCR 仪器的荧光信号采集通道数量所决定。现有的实时荧光 PCR 仪器通常可以同时检测 2-6 个不同荧光染料，因此，根据靶序列的数量，检测通道可以设计为 2-6 个。

[0016] 在本发明中，所述的荧光基团类型包括但不限于：ALEX-350、FAM、VIC、TET、CAL Fluor Gold 540、JOE、HEX、CAL Flour Orange 560、TAMRA、Cal Fluor Red 590、ROX、CAL Fluor Red 610、TEXAS RED、CAL Flour Red 635、Quasar 670、CY3、CY5、CY5.5 或 Quasar 705；用于以上荧光基团的淬灭基团包括但不限于 DABCYL、BHQ、ECLIPSE 或 TAMRA。

[0017] 在本发明中，同一检测通道相邻探针的 Tm 值的间隔差值可以是相同的或是不同的，不同检测通道探针的 Tm 值间隔差值可以是相同的或是不同的。

[0018] 在本发明中，Tm 值的间隔范围由实时荧光 PCR 仪器的熔解曲线分辨能力所决定。一般地，同一检测通道探针的 Tm 值的间隔差值可以为 1℃ -15℃。

[0019] 更佳地，同一检测通道探针的 Tm 值的间隔差值为 3℃ -8℃。

[0020] 在本发明中，较佳地，所述探针长度为 18bp 到 50bp。

[0021] 在本发明中，较佳地，探针 G+C 碱基的含量 30% -60%。

[0022] 本发明的技术原理如下：如图 1 所示，本发明首先在目标对象基因序列中选取相对保守的区域设计通用扩增引物。然后在引物扩增产物中选取差异度较大的区域设计能够识别各种目标核酸序列的特异探针，在探针设计过程中本发明通过调整探针的长度 (18bp 到 50bp) 和探针 G+C 碱基的含量 (30% 到 60%) 对每条探针的熔点温度 (Tm 值) 进行人为控制，使得同一检测通道内针对不同目标序列的检测探针 Tm 值都间隔合适的差值，Tm 值的

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间隔范围由实时荧光 PCR 仪器的熔解曲线分辨能力所决定。本发明在检测目标核酸序列时，特别增加一个检测通道用于检测体系内标的熔解曲线信号以保证体系的有效性。最后通过调整 PCR 扩增体系和 PCR 扩增程序中的各项条件，使得熔解曲线信号达到最佳本发明的另一目的是提供使用不同长度（或碱基组成）探针造成熔解曲线的 Tm 值差异达到单个荧光通 道多重检测的理念。

[0023] 本发明通过探针长度的变化调节探针熔解曲线的 Tm 值，使得每一个 Tm 值对应一个目标 DNA 序列，实现对目标序列的定性检测；通过定制每种目标序列的实时荧光 PCR 扩增 Ct 值工作曲线，实现对每个目标序列的定性检测；同时通过比较不同目标序列和探针产生的熔解曲线峰值高低，实现对多重目标序列间的比例判断。本发明通过实时荧光定量 PCR 技术与探针熔解曲线技术联用使得定性检测与定量检测有机结合。本发明根据探针熔解曲线峰值相对比较确定多重目标 DNA 序列间的比例关系。

[0024] 本发明技术具有以下优点：(1) 由于利用同一荧光染料标记的检测探针间 Tm 值的差异使得检测通量相对于传统实时荧光 PCR 技术提高了数倍，同时还保证了相同的检测灵敏度；(2) 由于单管中分别用 Tm 值和 Ct 值进行定性和定量的检测，该技术具有很高的检测准确率；(3) 由于能够单管同时平行检测多种型的靶序列，大大减少了检测技术的操作量和检测成本。(4) 该技术可以通过单管一次反应同时得到定性检测、定量检测和混合感染检测三重结果，而所需的检测时间与传统实时荧光 PCR 技术基本相同。(5) 该技术比起传统的荧光 PCR 技术具有良好的型间多态性位点耐受能力。

附图说明

[0025] 图 1 为本发明的技术原理图；

[0026] 图 2 为本发明实施例一的检测结果；

[0027] 图 3 为本发明实施例二的检测结果；

[0028] 图 4 为本发明实施例三的检测结果；

[0029] 图 5 为本发明实施例四的检测结果。

具体实施方式

[0030] 为了对本发明进行进一步的说明，我们以下实施例的方式进行阐述。以下实施例均使用 ABI7500 实时荧光 PCR 仪完成扩增曲线和熔解曲线的检测。

[0031] 检测体系：1*PCR buffer、2.5mM 镁离子、1U Taq DNA 聚合酶、200μM dNTP，1pmol 上游引物，20pmol 下游引物，5pmol 检测探针。

[0032] 检测程序：95℃预变性 3 分钟；95℃变性 10 秒，48℃退火 15 秒，72℃退火 20 秒，并于 48℃退火时检测荧光，重复 45 个循环；40℃ -85℃熔解曲线分析，每隔 1℃检测一次荧光信号。

[0033] 实施例一：

[0034] 单管三色体系定性检测 10 种 HPV 型别的阳性质粒标准品和内标基因。

[0035] 实验流程如下：

[0036] (1) 阳性质粒构建：根据 NCBI gene bank(http://www.ncbi.nlm.nih.gov) 上的 HPV-16，HPV-18，HPV-31，HPV-33，HPV-35，HPV-58，HPV-59，HPV-68，HPV-73，HPV-82 这 10 种

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HPV 基因型在 GP5+/GP6+ 引物（序列如下，GP5+ ：5’ -TTTGTTACTGTGGTAGATACTAC-3’，GP6+ ：5’ -GAAAAATAAACTGTAAATCATATTC-3’）扩增区内的 DNA 序列以及内标人源 β -globin 基因部分 DNA 序列进行 DNA 序列合成，并插入 pUC57 质粒载体的 SmaI 克隆位点中。10 种 HPV 型别的检测探针及相对于的 Tm 值温度如表一所示。

[0037] (2)PCR 扩增与熔解曲线分析检测 ：PCR 扩增及熔解曲线分析的体系如前面所述，每个反应体系中加入的标本量为 $1*10^6$ 拷贝 / 微升的不同型别的 HPV 标准品质粒及内标标准品质粒 5 微升，检测程序如前面所述。

[0038] (3) 结果判读 ：如图 1 所示，根据每种 HPV 型别在特定的检测通道特征 Tm 值即可判读样品中所携带的 HPV 型别 ；如果只有内标检测信号而没有 HPV 型别特征峰则说明样品中不携带这 10 种 HPV 型别 ；如果既没有内标检测信号也没有 HPV 型别检测信号则说明 PCR 扩增失败。

[0039] 如图 2 所示，该检测体系对 10 种 HPV 型别的都可检测出特异的熔解曲线检测信号，而且各个 HPV 型别间不存在交叉检出的现象，阴性对照中则只有内标 β -globin 基因的熔解曲线检测信号。

[0040] 表一 ：10 种 HPV 型别的检测探针及相对应的 Tm 值温度

[0041]

荧光基团	HPV 型别（内标）	探针序列（5’-3’）	T_m 值（℃）
	HPV-18	TGCTTCTACACAGTCTCCTGTACCTGGGCAATATGATGC	73.8

[0042]

ROX	HPV-35	TGTTCTGCTGTGTCTTCTAGTGACAGTACATATAAA	67.0
	HPV-16	ATTATGTGCTGCCATATCTACTTCAGAAA	63.0
	HPV-33	TATGCACACAAGTAACTAGTG	55.9
	HPV-31	GCAAACAGTGATACTACA	51.0
CY5	HPV-73	GTGTAGGTACACAGGCTAGTAGCTCTACTACAACGTATGCC	73.2
	HPV-59	TCTGTGTGTGCTTCTACTACTTCTTCTATTCCTAATG	67.5
	HPV-82	TTACTCCATCTGTTGCACAAACATTTAC	62.7
	HPV-58	TGAAGTAACTAAGGAAGGTACAT	58.2
	HPV-68	CTTTGTCTACTACTACTGAA	51.9
FAM	β-globin	GTTACAAGACAGGTTTAAGGAGACCAATAGA	64.7

[0043] 实施例二 ：

[0044] 单管三色体系 10 种 HPV 型别的阳性质粒标准品定量检测。

[0045] 引物及探针设计同实施例一。

[0046] (1)PCR 扩增检测 ：PCR 扩增的体系与程序如前面中所述，每个反应体系中加入的标本量为 $1*10^2$--$1*10^6$ 拷贝 / 微升的各种 HPV 型别的标准品质粒 5 微升。

[0047] (2) 工作曲线制作 ：如图 3 所示，根据各种 HPV 型别的标准品质粒梯度稀释后 PCR 扩增 Ct 值制作各种 HPV 型别相对应的定量检测工作曲线。

[0048] (3) 结果判读 ：在实施例一结果中判定 HPV 型别的基础上进一步通过样品 PCR 扩增 Ct 值在工作曲线上的对应位置判断样品中的病毒载量。

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[0049] 如图 3 所示，该检测体系在对 10 种 HPV 型别进行分型检测的同时还可进行定量分析。其中扩增曲线及工作曲线和 HPV 型别间的对应关系为：(A)HPV-18，(B)HPV-35，(C)HPV-16，(D)HPV-33，(E)HPV-31，(F)HPV-73，(G)HPV-59，(H)HPV-82，(I)HPV-58，(J)HPV-68。

[0050] 实时例三：

[0051] 引物及探针设计同实施例一。

[0052] HPV-16 与 HPV-18 不同比例 (0％，1％，3％，5％，10％，20％，30％，40％，50％，60％，70％，80％，90％，95％，97％，99％，100％) 的混合感染检测。

[0053] 实验流程如下：

[0054] (1)PCR 扩增检测：PCR 扩增及熔解曲线分析的体系与程序如前面所述，每个反应体系中加入的标本量为 $1*10^6$ 拷贝 / 微升的 HPV-16 和 HPV-18 混合标准品质粒 5 微升。

[0055] (2) 结果判读：如图 4 所示，通过 Tm 值范围判断病毒类型，通过峰值相对高低判断多重感染的病毒载量比例。

[0056] 如图 4 所示，该检测体系在对混合感染的检测能力下限为 3％。

[0057] 实施例四：9 种常见的蓟马的定性检测。

[0058] 实验流程如下：

[0059] (1) 阳性质粒构建：根据 NCBI gene bank(http://www.ncbi.nlm.nih.gov) 上的花蓟马、禾蓟马、西花蓟马、烟蓟马、棕榈蓟马、黄蓟马、黄胸蓟马、葱韭蓟马、普通大蓟马这 9 种蓟马 9 种蓟马线粒体基因的 COI(细胞色素氧化酵素) 区在 PF1/PR1(PF1：5'-ATTAGGAGC HCCHGAYATAGCATT-3'PR1：5'-CAGGCAAGATTAAAATATAAACTTGG-3') 引物扩增区内的目标 DNA 序列及内标 16SrDNA 基因部分 DNA 序列进行 DNA 序列合成并插入 pUC57 质粒载体的 SmaI 克隆位点中。

[0060] (2)PCR 扩增与熔解曲线分析检测：PCR 扩增及熔解曲线分析的体系如前面所述，每个反应体系中加入的标本量为 $1*10^6$ 拷贝 / 微升的不同型别的 HPV 标准品质粒及内标标准品质粒 5 微升，检测程序如发明内容中所述。

[0061] (3) 结果判读：如图 1 所示，根据每种蓟马在特定的检测通道特征 Tm 值即可判读样品所属的蓟马类型；如果只有内标检测信号而没有蓟马种类特征峰则说明样品不属于这 9 类的蓟马；如果既没有内标检测信号也没有蓟马种类检测信号则说明 PCR 扩增失败。

[0062] 注：1 = 花蓟马、2 = 禾蓟马、3 = 西花蓟马、4 = 烟蓟马、5 = 棕榈蓟马、6 = 黄蓟马、7 = 黄胸蓟马、8 = 葱韭蓟马、9 = 普通大蓟马、10 = 内标

[0063] 如图 5 所示，该检测体系对 9 种蓟马的都可检测出特异的熔解曲线检测信号，而且各个蓟马种间不存在交叉检出的现象，阴性对照中则只有内标的熔解曲线检测信号。

[0064] 9 种蓟马的检测探针及相对应的 Tm 值温度如表二所示。

[0065] 表二：9 种蓟马的检测探针及相对于的 Tm 值温度

[0066]

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荧光基团	蓟马种类（内标）	探针序列（5'-3'）	T_m 值（℃）
ROX	花蓟马	GAACAGTTTATCCACCATTATCAACATTCTATCATTCGGGTAC	70.5
	禾蓟马	TTATTTTAACTGCCAATTTTACTTCTTTTGTCTTTGCC	65.6
	西花蓟马	TCGGACCATCAGTAGATTTAACAATTTTTTT	62.1
	烟蓟马	TTTAACAGCCATTCTTCTTCTTTTAT	57.3
	棕榈蓟马	AGACTCAGATCCAGTACT	51.9
CY5	黄蓟马	TGATTTAACCAATTTTTTCATTACATTTAGCTGGAATCTCCTC	72.4
	黄胸蓟马	ACTCTATTAATTATAGGACTTATAAAAGAAGGAGCAGG	66.8
	葱韭蓟马	TTAGCAGGAGCTATCACTATACTTTTAACA	61.8
	普通大蓟马	AAAGGAAGGAGCAGCTAC	57.0
FAM	16S rDNA	AAAAGACGATAAGACCCTGTAGAA	60.6

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[0001]

说 明 书 核 苷 酸 和 氨 基 酸 序 列 表

<110> 厦门大学

<120>一种单管定性并定量检测多个目标核酸序列的方法

<160> 21

<210> 1
<211> 29
<212> DNA
<213> 人工序列

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<210> 2
<211> 26
<212> DNA
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<210> 3
<211> 29
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attatgtgct gccatatcta cttcagaaa 29

<210> 4
<211> 21
<212> DNA
<213> 人工序列

<400> 4
aatgcacaca agtaactagt g 21

<210> 5
<211> 18
<212> DNA

[0002]

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<213> 人工序列

<400> 5
gcaaacagtg atactaca 18

<210> 6
<211> 41
<212> DNA
<213> 人工序列

<400> 6
gtgtaggtac acaggctagt agctctacta caacgtatgc c 41

<210> 7
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<400> 7
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<210> 8
<211> 19
<212> DNA
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<400> 8
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<210> 9
<211> 23
<212> DNA
<213> 人工序列

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tgaagtaact aaggaaggta cat 23

<210> 10
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<212> DNA

[0003]

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<213> 人工序列

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ctttgtctac tactactgaa 20

<210> 11
<211> 31
<212> DNA
<213> 人工序列

<400> 11
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<213> 人工序列

<400> 12
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<210> 13
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<210> 14
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<210> 15
<211> 26
<212> DNA
<213> 人工序列

[0004]

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<400> 15
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<210> 16
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<212> DNA
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agactcagat ccagtact 18

<210> 17
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<212> DNA
<213> 人工序列

<400> 17
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<210> 18
<211> 20
<212> DNA
<213> 人工序列

<400> 18
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<211> 20
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<210> 20
<211> 20
<212> DNA
<213> 人工序列

[0005]

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<400> 20
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<210> 21
<211> 20
<212> DNA
<213> 人工序列

<400> 21
aaaagacgat aagaccctgt agaa 24

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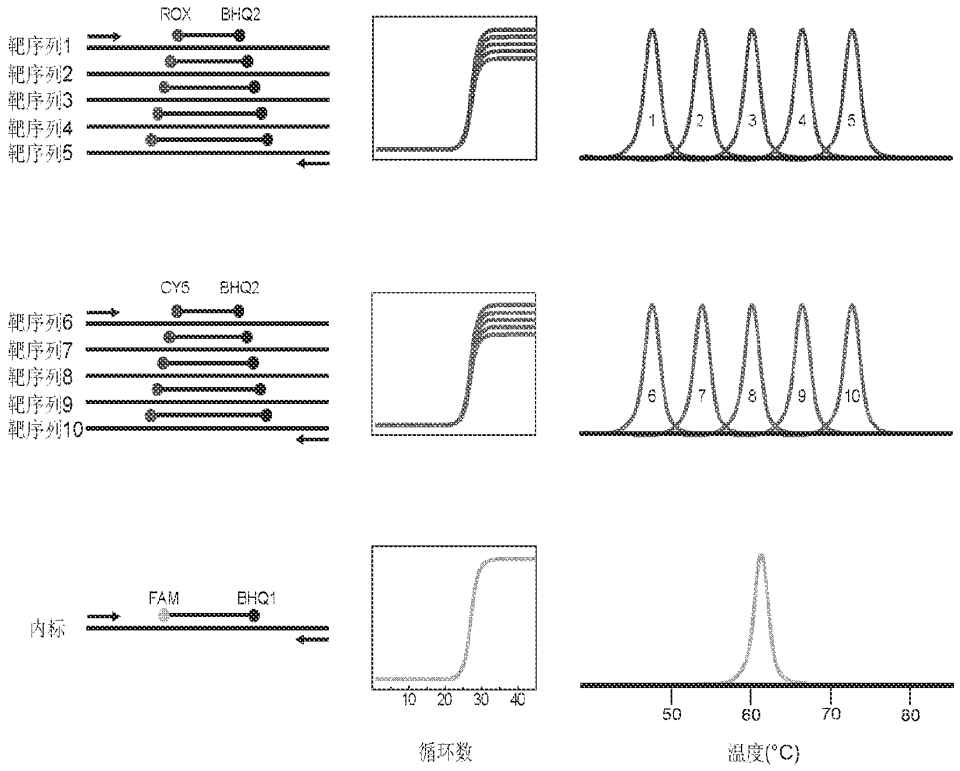


图 1

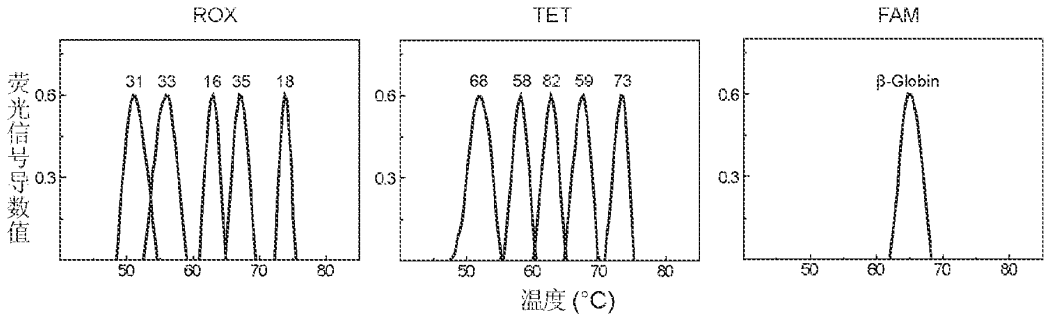


图 2

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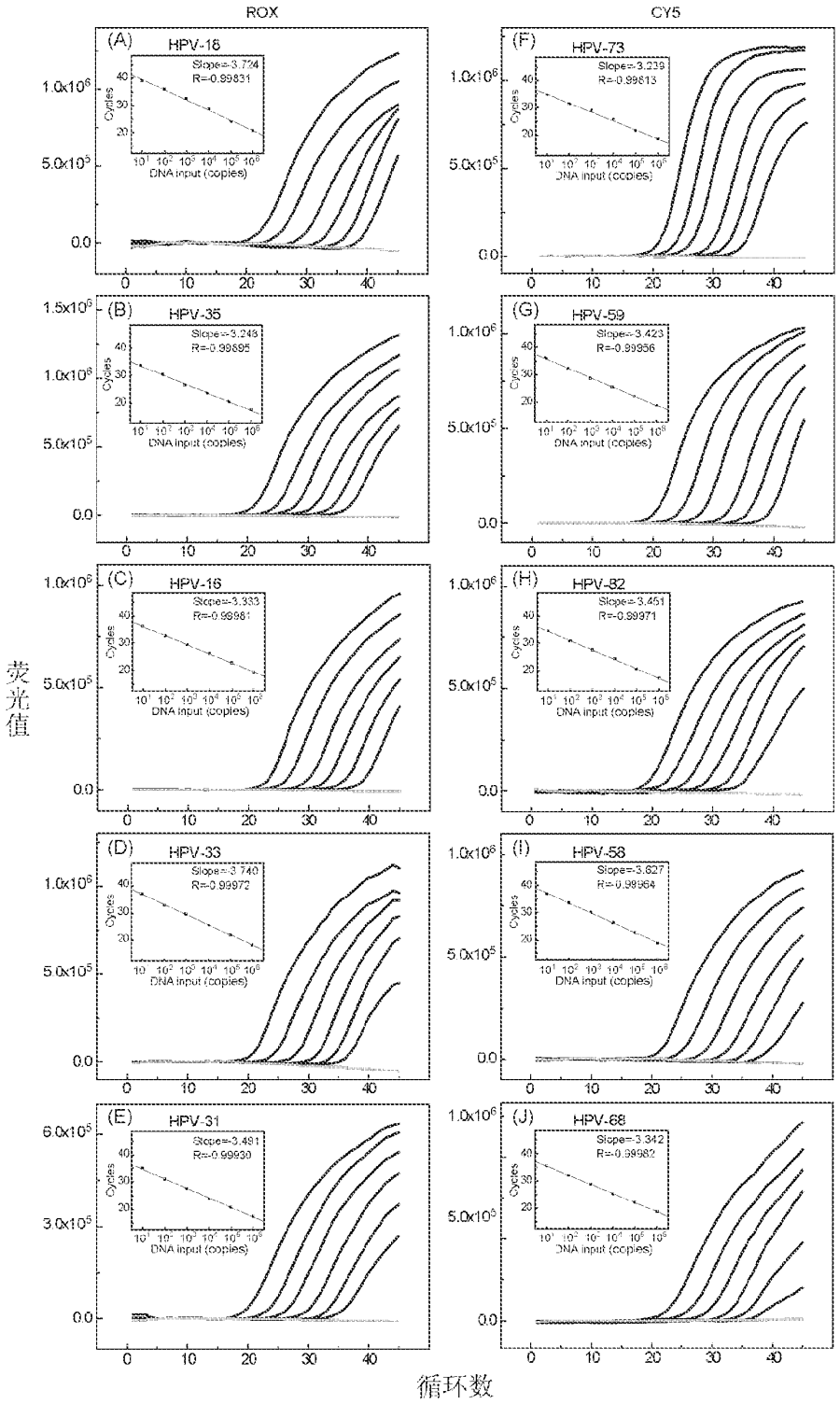


图 3

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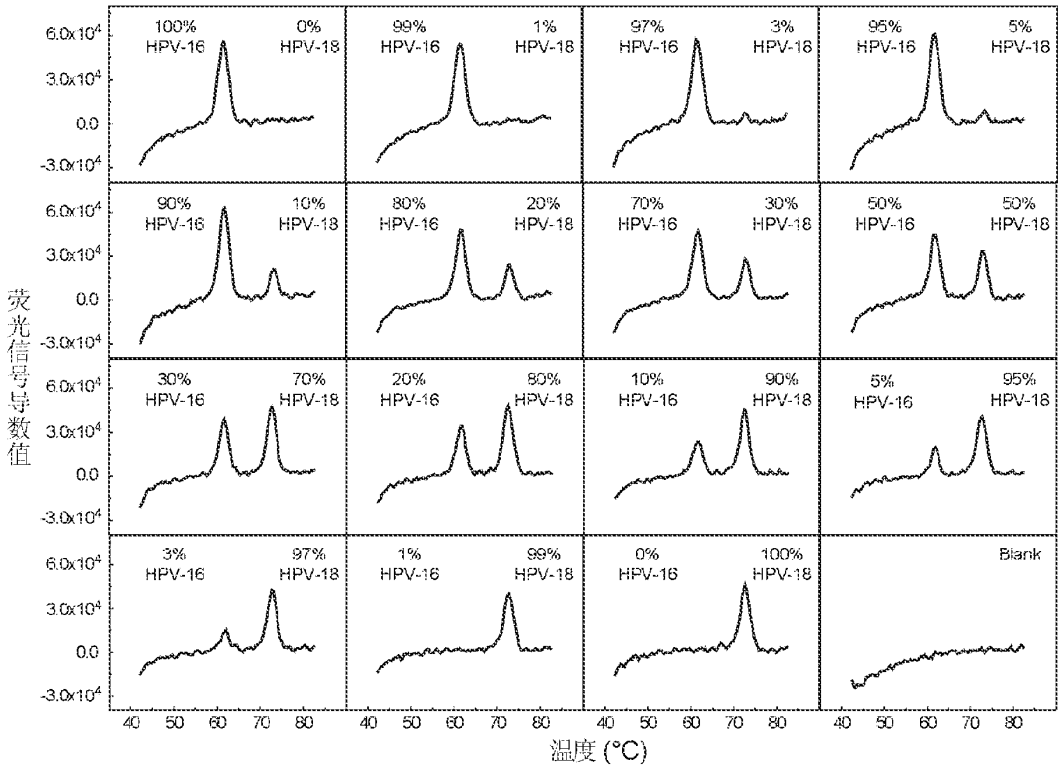


图 4

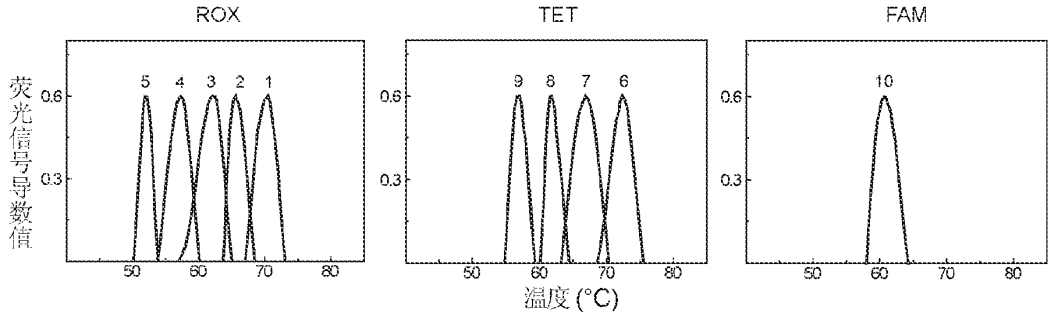


图 5

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UNITED STATES
PATENT AND TRADEMARK OFFICE

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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/352,112	**11/12/2024 08:03:34 PM Z ET**	**88DG-373535-US**

Title of Invention

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	3145	FILED BY	Annie Wong
PATENT CENTER #	67939458	FILING DATE	07/13/2023
CUSTOMER #	30764	FIRST NAMED INVENTOR	Emil P. KARTALOV
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	David Heisey

Documents

TOTAL DOCUMENTS: 4

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
IDS-5_18352112.pdf	4	Information Disclosure Statement (IDS) Form (SB08)	598 KB
CN102559868A.pdf	28	Foreign Reference	4576 KB
WO2004-099434A2.pdf	37	Foreign Reference	1650 KB
SR2_CN2022103893474_12 Oct2024.pdf	2	Other reference-Patent/Application/Search Documents	114 KB

Digest

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IDS-5_18352112.pdf	D9A2EF3A12DC33E8EA0F7DF54F992D7B6B129B5CC6A44AE5

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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 filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

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

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

New International Application Filed with the USPTO as a Receiving Office

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

Attorney Docket No.: 88DG-373535-US
CIT-6088-US6

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION

Title:

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

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Attorney Docket No.: 88DG-373535-US (CIT-6088-US6)

Sheets of Drawings: 8

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of U.S. Application No. 16/937,464, filed on July 23, 2020, which is a continuation of U.S. Application No. 15/914,356, filed on March 7, 2018, issued as U.S. Patent No. 10,770,170, which is a continuation of U.S. Application No. 14/451,876, filed on August 5, 2014, issued as U.S. Patent No. 10,068,051, which is a continuation of U.S. Application No. 13/756,760, filed on February 1, 2013, issued as U.S. Patent No. 8,838,394, which claims the benefit of U.S. provisional applications 61/594,480, filed February 3, 2012 and 61/703,093, filed September 19, 2012, each of which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

[0002] This application contains a Sequence Listing which has been submitted electronically in XML format and is incorporated by reference in its entirety. The XML copy, created on July 12, 2023, is named 373535.xml and is 57,570 bytes in size.

GOVERNMENT RIGHTS

[0003] This invention was made with government support under Grant No. 1144469 awarded by the National Science Foundation. The government has certain rights in the invention.

BACKGROUND

[0004] Multiplexed reactions offer significant advantages over traditional uniplex reactions, including performance of parallel reactions on the same sample, use of the same chamber to perform multiple reactions, and the ability to extract rich information from a sample in a fast and efficient manner. However, to achieve these benefits, multiplexed assays generally require complex reporting mechanisms, namely spectrally resolved fluorescence or chemiluminescence (e.g., PCR, ELISA), spatially resolved signals (e.g., microarrays, gel electrophoresis), temporally resolved signals (e.g., capillary electrophoresis), or combinations thereof (e.g., Sanger

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sequencing). There is a need for multiplexed reactions that can be carried out in a single solution.

SUMMARY OF THE INVENTION

[0005] This disclosure provides methods, compositions, systems, and kits for the multiplexed detection of analytes. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some examples, each of the analytes is encoded as a value of one (or at least one) component of a signal.

[0006] In some examples, an assay provided in this disclosure is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = \log_2 (F + 1)$ and F is the maximum cumulative value of one component of a signal (e.g., an intensity) when all the analytes are present.

[0007] In some cases, each analyte is encoded as at least one first value in a first component of a signal (e.g., at least one intensity or range of intensities) and at least one second value in a second component of a signal (e.g., at least one wavelength or range of wavelengths). In some examples, each analyte is encoded as a first value in a first component of a signal at each of a plurality of second values in a second component of a signal (e.g., a signal intensity or range of signal intensities at each of a plurality of wavelengths or ranges of wavelengths).

[0008] In some examples, an assay provided herein is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = C * \log_2 (F + 1)$, where C is the number of the second values used to encode the analytes and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present.

[0009] In some cases, an assay provided in this disclosure is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = (P * T) + 1$, where P is the number of codes per tier in a coding scheme and T is the number of tiers. In some cases, the number of tiers $T = \log_4 (F + 1)$, and F is the maximum cumulative value of a first component of a signal, for any second value, when all of the analytes are present.

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[0010] In some examples, the first value is an intensity or range of intensities. In some cases, a coding scheme comprises at least three intensities or ranges of intensities.

[0011] In some cases, the second value is a wavelength or range of wavelengths. In some examples, the coding scheme comprises at least five wavelengths or ranges of wavelengths.

[0012] In some examples, the signal is an electromagnetic signal. In some cases, the electromagnetic signal is a fluorescence emission signal. In some examples, the intensity of the fluorescence emission signal is measured at at least four wavelengths or ranges of wavelengths.

[0013] In some cases, an assay provided herein is performed with reagents that are lyophilized prior to use.

[0014] In some cases, a detecting step is performed with reagents comprising hybridization probes. In some examples, the number of the hybridization probes is greater than the number of analytes. In some cases, the set of hybridization probes comprise one or more hybridization probes specific for different analytes and comprising an identical fluorophore or combination of fluorophores. In some examples, a sample is contacted with at least 18 of said hybridization probes. In some examples, a detecting step is performed with reagents comprising at least one pair of primers. In some cases, at least one pair of primers are capable of amplifying a region complementary to at least three of said hybridization probes.

[0015] In some examples, the signal that is measured is generated during a polymerase chain reaction. In some cases, the polymerase chain reaction is selected from the group consisting of an end-point polymerase chain reaction, a real-time polymerase chain reaction, a digital polymerase chain reaction, and combinations thereof.

[0016] In some examples, at least one cumulative measurement is performed on a solution.

[0017] In some examples, at least one analyte is encoded by at least one additional value (i.e., at least two values together), wherein the at least one additional value is selected from the group consisting of a value from at least one additional component of a signal, a value from at least one component of a different signal, and combinations thereof. For example, the at least one additional value may be selected from the group consisting of a fluorescence emission intensity, a fluorescence emission wavelength, a Förster resonance energy transfer (FRET) emission intensity, a FRET emission wavelength, an electrochemical signal, a chemiluminescence

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wavelength, a chemiluminescence intensity, a fluorescence bleaching rate, a chemiluminescence bleaching rate, and combinations thereof.

[0018] In some cases, a chromatogram is constructed. The chromatogram may be constructed by plotting all possible combinations of first values and second values for positive control samples for each analyte.

[0019] In some examples, at least one of the analytes comprises a polynucleotide. In some cases, the polynucleotide is from a source selected from the group consisting of an animal, a plant, a bacteria, a fungus, a parasite, and a virus. In some examples, the polynucleotide is from a source selected from the group consisting of human immunodeficiency virus, herpes simplex virus, human papilloma virus, Plasmodium, Mycobacterium, dengue virus, hepatitis virus, and influenza virus. In some cases, the polynucleotide is selected from the group consisting of human immunodeficiency virus polyprotease, human immunodeficiency virus p17, human papilloma virus E6, and human papilloma virus E7.

[0020] In some cases, a sample is selected from the group consisting of a clinical sample, a food sample, an environmental sample, a pharmaceutical sample, and a sample from a consumer product.

[0021] In some examples, information concerning the presence or absence of an analyte is transmitted through a computer network.

[0022] In some cases, information concerning the presence or absence of an analyte is provided to a physician. In some examples, a clinical decision is made based on such information.

[0023] In some examples, at least one step of a method provided herein is performed using instructions on a computer readable medium. In some cases, the instructions are located on a remote server. In some examples, the instructions are located on a thermal cycler. In some cases, the instructions are located on a computer in communication with a thermal cycler.

[0024] In some cases, at least one of the analytes is a positive control analyte.

[0025] In some examples, the coding scheme is non-degenerate. In some cases, the coding scheme is designed to be non-degenerate. In some examples, the coding scheme is made non-degenerate by enumerating every legitimate result that can be obtained from the coding scheme, identifying each legitimate result that is degenerate, and eliminating at least one potential analyte code from the coding scheme to eliminate degeneracy.

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[0026] In some cases, an assay provided herein is an end-point assay. In some examples an assay provided in this disclosure is ended at a threshold number of cycles set by the limit of detection of an instrument.

[0027] In certain examples, an assay provided herein is a liquid phase assay.

[0028] In some cases, an assay provided herein is quantitative.

[0029] In some cases, this disclosure provides a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

[0030] In some examples, the method described in the preceding paragraph is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = \log_2 (F + 1)$ and F is the maximum cumulative value of the first values when all of the analytes are present. In some cases, the first value is an intensity or range of intensities. In some examples, the first value has a minimum value in the coding scheme that is selected from the group consisting of at least 1, at least 2, at least 4, at least 8, at least 16, at least 32, and at least 64. In some cases, the first value is incremented in the coding scheme by an amount equal to the cumulative maximum of said preceding first values plus one. In other cases, the first value is incremented in the coding scheme by an amount greater than the cumulative maximum of the preceding first values plus one.

[0031] In some cases, this disclosure provides a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding

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scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said at least one first value and at least one said second value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme, wherein, when degeneracy is eliminated, said method is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

[0032] In some cases, each of the analytes of the method described in the preceding paragraph is encoded as a first value in a first component of the signal at each of a plurality of second values in a second component of the signal. In some cases, when degeneracy is eliminated, this method is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single volume using four second values. In some examples, when degeneracy is eliminated, the coding scheme comprises T non-degenerate tiers, wherein $T = \log_4 (F + 1)$ and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present. In some cases, when degeneracy is eliminated, the method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = (P * T) + 1$ and P is the number of codes per tier. In some examples, when degeneracy is eliminated, the method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = C * \log_2 (F + 1)$, C is the number of second values in the coding scheme, and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present.

[0033] This disclosure also provides non-degenerate coding schemes capable of unambiguously encoding the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis.

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[0034] In some examples, a non-degenerate coding scheme of this disclosure is generated by a method comprising: (a) generating a code for each potential analyte, wherein each potential analyte is encoded by at least one value of at least one component of a signal; (b) enumerating every legitimate cumulative result for all possible combinations of presence or absence of each analyte; (c) identifying each legitimate result that is degenerate; and (d) eliminating at least one code to eliminate degeneracy. In some cases, the coding scheme may be expanded by adding additional codes that are at least one unit greater than the sum of all previous codes in the at least one component of said signal. In some cases a coding scheme is generated by a method of mathematical iteration that guarantees non-degeneracy by construction.

[0035] In some cases, this disclosure provides systems for detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

[0036] In some cases, this disclosure provides systems for detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values and said second values within said sample, thereby

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providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme, wherein, when degeneracy is eliminated, said system is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

[0037] In some cases, this disclosure provides methods of detecting the presence or absence of each analyte of a plurality of analytes for a third party, comprising: (a) obtaining the identity of each of said analytes from a party; (b) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (c) providing said party with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present, said party: (i) contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and (ii) cumulatively measuring said first values within said sample, thereby providing a cumulative measurement; and (d) obtaining said cumulative measurement from said party; (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and (f) providing said party with information about the presence or absence of each of said analytes.

[0038] In some cases, this disclosure provides methods of detecting the presence or absence of each analyte of a plurality of analytes for a third party, comprising: (a) obtaining the identity of each of said analytes from a party; (b) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (c) providing said party with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present, said party: (i) contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and (ii) cumulatively measuring said first values and said second values within said

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sample, thereby providing a cumulative measurement; and (d) obtaining said cumulative measurement from said party; (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and (f) providing said party with information about the presence or absence of each of said analytes.

[0039] In some cases, this disclosure provides compositions for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising a first value, wherein said reagents are capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

[0040] In some cases, this disclosure provides compositions for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said reagents are capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

[0041] In some cases, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising a first value, wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

[0042] In some examples, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

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[0043] In some cases, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising a kit body (601), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (602), a bottle comprising primers for amplification (603), and a bottle comprising reagents for amplification (604), wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

[0044] In some examples, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, a kit body (601), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (602), a bottle comprising primers for amplification (603), and a bottle comprising reagents for amplification (604), each probe generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said probes generates only one second value.

INCORPORATION BY REFERENCE

[0045] 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.

BRIEF DESCRIPTION OF THE DRAWINGS

[0046] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0047] Fig. 1 shows a comparison between a traditional encoding method of detecting four analytes with four colors and an encoding method of the invention able to detect 16 or more sequences with four colors. Colors are indicated by B, G, Y, and R, which indicate blue, green, yellow, and red, respectively.

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[0048] Fig. 2 shows a schematic representation of detection of six analytes (including control) with four colors. The analytes are detected using hybridization probes attached to a fluorophore (B, G, Y, R; blue, green, yellow, and red, respectively) and a quencher (oval with "X"). The control sequence and the five other sequences (Dengue Fever = dengue virus; Tuberculosis = *Mycobacterium tuberculosis*; P17 = HIV p17; Malaria = *Plasmodium falciparum;* and Herpes = herpes simplex virus 2) are all detected using a probe labeled with a blue fluorophore. The non-control analytes are each detected using 1-3 additional probes. For example, the dengue virus analyte is detected using three additional probes with green, yellow, and red fluorophores; the herpes simplex virus 2 analyte is detected using one additional probe with a red fluorophore; and so on.

[0049] Fig. 3 shows chromatograms of experimental results obtained as described in Example 2.

[0050] Fig. 4 shows an exemplary embodiment of the invention in which a computer is used to perform one or more steps of the methods provided herein.

[0051] ~~**Fig. 5** shows a schematic~~ **Figs. 5A** and **5B** show schematics of two different methods of detecting wavelength and intensity of a light signal, such as a fluorescence emission signal.

[0052] Fig. 6 shows a schematic of exemplary kits.

[0053] Fig. 7 shows chromatograms of experimental results obtained as described in Example 3.

[0054] Fig. 8 shows a schematic of a legitimate result (top) and an illegitimate result (bottom).

DETAILED DESCRIPTION OF THE INVENTION

[0055] Fluorescence detection has been a preferred technique for multiplexed assays because of several desirable features, including compatibility with biochemical assays, the relatively small size of fluorescent labels, simple means of conjugation to molecules of interest, affordability, low toxicity, stability, robustness, detectability with inexpensive optics, and an ability to be combined with spatial arrays. However, standard fluorophores have wide emission spectra. Therefore, in order to avoid spectral overlap (i.e., to preserve spectral resolution) only a relatively small number of colors (e.g., 4 to 6) are typically used simultaneously in multiplexed fluorescent assays.

[0056] The traditional encoding method for multiplexed fluorescent assays has been to encode each analyte with a single color; i.e., M=N, where M is the number of analytes that can be

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detected and N is the number of spectrally resolved fluorescent probes. Whenever higher factors of multiplexing are required (i.e., M>N), fluorescence is generally combined with other techniques, such as aliquoting, spatial arraying, or sequential processing. These additional processing steps are labor-intensive and frequently require relatively expensive and complex optical and mechanical systems (e.g., spectrometers, mechanized microscopy stages, microfluidics, droplet generators, scanners, and the like). Such systems are often impractical to deploy in certain settings, particularly point-of care and low-resource settings. Thus, there is a significant need for multiplexed encoding and decoding methods that can provide an inexpensive means of multiplexing while avoiding the use of expensive additional processing steps.

[0057] This disclosure provides methods, systems, compositions, and kits for the detection of multiple analytes in a sample. Analytes are detected based on the encoding, analysis, and decoding methods presented herein. In some examples, each analyte to be detected is encoded as a value of a signal (e.g., intensity), where the values are assigned so that the results of the assay unambiguously indicate the presence or absence of the analytes being assayed. In other examples, each analyte to be detected is encoded as a value in each of at least two components of a signal (e.g., intensity and wavelength). The at least two components of a signal may be orthogonal. Similarly, as described more fully elsewhere in this disclosure, multiple orthogonal signals may be used, such as a combination of a fluorescent signal and an electrochemical signal. The analyte may be any suitable analyte, such as a polynucleotide, a protein, a small molecule, a lipid, a carbohydrate, or mixtures thereof. The signal may be any suitable signal such as an electromagnetic signal, a light signal, a fluorescence emission signal, an electrochemical signal, a chemiluminescent signal, and combinations thereof. The at least two components of the signal may be any suitable two components, such as an amplitude and a frequency or an intensity and a wavelength.

[0058] After encoding of the analytes, a sample is provided wherein the sample comprises or may comprise at least one of the encoded analytes. The sample is contacted with an analyte-specific reagent or reagents that generate a particular signal, as specified for each analyte in the coding scheme, in the presence of an analyte. A reagent may be any suitable reagent that is capable of generating such a signal in the presence of its corresponding analyte, for example, an oligonucleotide probe attached to a fluorophore and a quencher (e.g., a TAQMAN probe). If the

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reagent is an oligonucleotide probe attached to a fluorophore and a quencher, a nucleic acid amplification may be performed to generate the signal.

[0059] After addition of the reagent(s), the signal is quantified. In some cases, this quantification is performed by measuring one component of the signal (e.g., fluorescence intensity) and determining the presence and absence of certain analytes based on the values used to encode the presence of each analyte and the cumulative value of the signal.

[0060] In some cases, at least two components of a signal (e.g., intensity and wavelength) are cumulatively measured for the sample. This measurement can be performed, for example, by measuring the intensity at a particular wavelength or the intensity within a particular range of wavelengths. The presence or absence of an analyte may then be determined based on the values of each of the at least two components of the signal and the values used to encode the presence of the analyte (i.e., those values in the coding scheme).

[0061] The encoding may be performed in a manner that reduces or eliminates the number of possible degenerate (e.g., ambiguous) results that can be obtained by the method. As described elsewhere in this specification, the full coding capability of a particular coding scheme may be enumerated, and certain potential analyte codes may be eliminated from the coding scheme to reduce or eliminate degeneracy. Similarly, a coding scheme may be designed to be non-degenerate, so that a reduction or elimination of degeneracy is not necessary. A decoding matrix may be constructed to translate cumulative signal measurements (e.g., intensities or intensities at particular wavelengths) into the presence or absence of certain analytes, corresponding to the constituent signals of the cumulative signal measurement.

I. Definitions

[0062] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

[0063] As used herein, the singular forms “a,” “an,” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms “including,” “includes,” “having,” “has,” “with,” “such as,” or variants thereof, are used in either the specification and/or the claims, such terms are not limiting and are intended to be inclusive in a manner similar to the term “comprising”.

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[0064] The term "about," as used herein, generally refers to a range that is 15% greater than or less than a stated numerical value within the context of the particular usage. For example, "about 10" would include a range from 8.5 to 11.5.

[0065] The terms "dimension" and "component," as used herein when referring to a signal, generally refer to an aspect of the signal that may be quantified. For example, if a signal is generated by a fluorescent molecule, it may be quantified in terms of its wavelength (e.g., a first dimension or component) and its intensity (e.g., a second dimension or component).

[0066] The term "decoding," as used herein, generally refers to a method of determining which analytes are present based on the cumulative signal and a coding scheme or decoding matrix that enables the conversion of a cumulative signal to information concerning the presence or absence of one or more analytes.

[0067] The term "encoding," as used herein, generally refers to the process of representing an analyte using a code comprising values of a signal, such as intensity, or values in each of at least two components of a signal or signals, such as wavelength and intensity.

[0068] The terms "oligonucleotide probe attached to a fluorophore and a quencher" and "TAQMAN probe" generally refer to hydrolysis probes used to detect the presence of an analyte in a polynucleotide amplification assay. These probes comprise an oligonucleotide probe attached to a fluorophore and a quencher. So long as the quencher and the fluorophore are in proximity, the quencher quenches the fluorescence emitted by the fluorophore upon excitation by a light source. The sequence of the oligonucleotide probe is designed to be complementary to a polynucleotide sequence present in an analyte, and therefore capable of hybridizing to the polynucleotide sequence present in the analyte. Hybridization of the oligonucleotide probe is performed in a nucleic acid amplification reaction comprising primers (e.g., a polymerase chain reaction). Upon extension of the primers by a DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and the quencher into the medium. The proximity between the fluorophore and the quencher is broken and the signal from the fluorophore is no longer quenched. Thus, the amount of fluorescence detected is a function of the amount of analyte present. If no analyte is present, the probe will not hybridize to an analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be produced.

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[0069] The term "orthogonal," as used herein, generally refers to at least two components of a signal (e.g., wavelength and intensity), or at least two different signals (e.g., fluorescence emission and electrochemical signal), that can be varied independently or approximately independently. For example, wavelength and intensity are considered orthogonal or approximately orthogonal when fluorescent molecules are used. Among other factors, the wavelength of fluorescence emission will depend on the composition of the fluorescent molecule and the intensity of the fluorescence will depend on the amount of molecule present. Although wavelength and intensity are examples of two components of a signal that can be varied approximately independently, the methods described herein are not limited to components that can be varied independently or approximately independently. Components of a signal, or signals, that vary non-independently may also be used, so long as the components or signals are characterized well enough to enable encoding, measurement, and decoding. For example, if the variance in one component or signal affects the variance in another component or signal, the two components or signals may still be used so long as the relationship between the variances is understood.

[0070] The terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides. Exemplary polynucleotides include deoxyribonucleic acids, ribonucleic acids, and synthetic analogues thereof, including peptide nucleic acids.

[0071] The term "probe," as used herein, generally refers to a reagent capable of generating a signal in the presence of a particular analyte. A probe generally has at least two portions: a portion capable of specifically recognizing an analyte, or a portion thereof, and a portion capable of generating a signal in the presence of an analyte, or a portion thereof. A probe may be an oligonucleotide probe attached to a fluorophore and a quencher, as described above and elsewhere in this disclosure. A probe may also be any reagent that generates a signal in the presence of an analyte, such as an antibody that detects an analyte, with a fluorescent label that emits or is quenched upon binding of the antibody to an analyte. Any suitable probe may be used with the methods presented in this disclosure, so long as the probe generates a quantifiable signal in the presence of an analyte. For example, the analyte-specific portion of a probe may be coupled to an enzyme that, in the presence of an analyte, converts an uncharged substrate into a

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charged product, thereby increasing the electrical conductivity in the medium over time. In this case, different analytes may be encoded by coupling the analyte-specific portion of the probe (e.g., hybridization probe or antibody) to an enzyme at different ratios. The resulting rate of increased conductivity in the medium will be cumulative for all analytes present in the medium. Encoding analytes according to the methods provided herein enables conversion of the conductivity measurements into unambiguous (i.e., non-degenerate) results providing information about the presence or absence of particular analytes. Similarly, a probe may comprise an enzyme producing a chemiluminescent product from a substrate. The amount of chemiluminescence may then be used to encode the presence of particular analytes.

II. Encoding and Decoding Methods

A. Traditional Fluorescent Encoding and Decoding Method

[0072] A commonly used method of determining the presence of an analyte uses four spectrally resolved fluorescent molecules to indicate the presence or absence of four analytes. An example of this method is presented on the left-hand side of **Fig. 1**. The left-hand side of **Fig. 1** shows an encoding method where four analytes (Seq 1, Seq 2, Seq 3, and Seq 4) are each encoded by a single color (blue, green, yellow, and red, respectively). The color represents a fluorophore attached to an oligonucleotide probe that also comprises a quencher. In the system shown on the left-hand side of **Fig. 1**, there are four different oligonucleotide probes, each comprising a single fluorophore (blue, green, yellow, or red) and a quencher. The presence or absence of an analyte is determined based on the presence or absence of a signal in a particular color.

[0073] The chart on the left-hand side of **Fig. 1** shows intensity versus color for a hypothetical sample containing two analytes: Seq 1 and Seq 3. The presence of these analytes is determined based on the measurement of a blue signal (corresponding to Seq 1) and a yellow signal (corresponding to Seq 3). The absence of Seq 2 and Seq 4 is indicated by the absence of a blue and red signal.

[0074] Table 1 shows a translation of this coding scheme into a binary format. Each analyte is encoded as a value in each of two components of a fluorescent signal: (1) color (also known as wavelength; or range of wavelengths) and (2) intensity (indicated by the numbers within the table). For example, A (e.g., Seq 1) has a color of blue and an intensity of 1; B (e.g., Seq 2) has

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a color of green and an intensity of 1; and so on. The intensity of the signal within each color range may be quantified as described herein, for example by measuring the intensity of the signal within a particular wavelength range determined by a band pass filter. A result of 1000 indicates that only analyte A is present; a result of 1100 indicates that analytes A and B are present; and so on.

Table 1. Traditional encoding of four analytes with four probes, each probe having a single color, and one probe per analyte.

	Blue	**Green**	**Yellow**	**Red**
A	1	0	0	0
B	0	1	0	0
C	0	0	1	0
D	0	0	0	1

B. Encoding Methods Using More Than One Color Per Analyte

[0075] The traditional method described above suffers from the fact that it is limited by the number of spectrally resolvable fluorophores. More specifically, the number of detectable analytes is equal to the number of spectrally resolvable fluorophores. Therefore, the number of analytes may only be increased by increasing the number of spectrally resolvable fluorophores.

[0076] This disclosure provides methods that overcome this limitation. More specifically, in some cases, by utilizing at least two components of a signal during encoding, the methods described herein may be used to detect more than one analyte per fluorophore. For example, using the method provided herein 1.5, 1.6, 1.7, 1.8, 1.9, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes may be detected per fluorophore. In some cases, the methods provided herein may be used to detect at least 1.5, 1.6, 1.7, 1.8, 1.9, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes may be detected per fluorophore. In some cases, the methods provided herein may be used to detect 1.5-2, 2-4, 1.5-4, 4-6, 2-6, 6-10 analytes per fluorophore.

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[0077] In some cases, the methods provided in this disclosure may include the use of a control color. The control color may be attached to one or more probes binding a positive control analyte, and each analyte to be detected, in a sample. If the same sequence occurs in the positive control analyte and each analyte to be detected, a single control probe may be used. If the same sequence does not occur in the positive control analyte and each analyte to be detected, different probes may be used, but each probe may still be attached to the control color.

[0078] For example, building on the traditional methods described above, one color (e.g., blue) may be used to encode the presence of a control analyte that is always present in the sample. The control analyte may be added to the sample, or may be inherently present in the sample. The remaining colors (e.g., green, yellow, red) may be used to encode the presence of additional analytes. Table 2 shows an example of one such method.

Table 2. Encoding of four analytes, including control, with four colors and up to two colors per analyte.

	Blue	**Green**	**Yellow**	**Red**
Ctrl. (p)	1	0	0	0
A	1	0	0	1
B	1	0	1	0
C	1	1	0	0

[0079] In Table 2, the presence of the control (p) is indicated by a result of 1000. The presence of the control and analyte A is indicated by a result of 2001. The presence of the control and all three other analytes is indicated by a result of 4111. The control color (blue) provides an indication that the assay is functioning properly. The intensity of the blue color reports the number of analytes that are present in a test sample. Of course, any color may be used as the control color. One of skill in the art will recognize that certain practical considerations might make it preferable to use one color over another as the control color. For example, if one color is better detected in a particular optical system (or system of fluorophores), it might be practically preferable to use that color as a control color.

[0080] The coding scheme shown in Table 2 encodes each analyte using one control color and one additional color (up to 2 probes per analyte). However, as shown below, the number of

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analytes that can be encoded increases when up to 4 colors are used per analyte. Table 3 shows an exemplary coding scheme where each analyte is encoded by up to 4 colors. In the scheme shown in Table 3, the control color (blue) is generated in the presence of the control sequence and each of the other seven analytes (A-G). The other seven analytes are each encoded by the presence of one to three additional colors. The colors may be contained on different probes (e.g., oligonucleotide probes attached to a fluorophore and a quencher) or on the same probe (which can have multiple fluorophores and multiple quenchers).

Table 3. Encoding of eight analytes (including control) with four colors and up to four colors per analyte.

	Blue	**Green**	**Yellow**	**Red**
Ctrl. (p)	1	0	0	0
A	1	0	0	1
B	1	0	1	0
C	1	1	0	0
D	1	0	1	1
E	1	1	0	1
F	1	1	1	0
G	1	1	1	1

[0081] In Table 3, the presence of the control and all seven other analytes is indicated by a result of 8444. Three analytes (A, B, and C) are encoded by two colors. Three analytes (D, E, and F) are encoded by three colors, and one analyte (G) is encoded by four colors. The presence of the control (p) is indicated by a result of 1000. The presence of the control and analyte A is indicated by a result of 2001. The presence of the control and analyte G is indicated by a result of 2111. These results express the cumulative intensity of the signal in each color. For example, the result 2111 has a 2X signal intensity in the blue channel, while the result 1000 has only a 1X signal intensity in the blue channel.

[0082] Using Table 3, each of the possible cumulative assay results can be enumerated, in terms of color (blue, green, yellow, red) and intensity (0-8). Table 4 shows a "decoding matrix" generated by enumerating each of the possible cumulative assay results based on the encoding method presented in Table 3 and providing the corresponding decoded result of each assay in terms of the analyte(s) present in the sample. A similar decoding matrix may be generated for

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any coding scheme described herein, by enumerating each of the possible cumulative assay results based on the coding scheme and providing the corresponding decoded result of each assay in terms of the analyte(s) present in (or absent from) the sample. In some cases, as described below, one or more analytes may be removed from the coding scheme in order to reduce or eliminate degeneracy.

Table 4. Decoding matrix for encoding method presented in Table 3.

Cumulative Assay Result				
Blue	**Green**	**Yellow**	**Red**	**Analyte(s) Present**
1	0	0	0	p
2	0	0	1	pA
2	0	1	0	pB
2	1	0	0	pC
2	0	1	1	pD
2	1	0	1	pE
2	1	1	0	pF
2	1	1	1	pG
3	0	1	1	pAB
3	1	0	1	pAC
3	0	1	2	pAD
3	1	0	2	pAE
3	1	1	1	pAF, pCD, pBE
3	1	1	2	pAG, pDE
3	1	1	0	pBC
3	0	2	1	pBD
3	1	2	0	pBF
3	1	2	1	pBG, pDF
3	2	0	1	pCE
3	2	1	0	pCF
3	2	1	1	pCG, pEF
3	1	2	2	pDG
3	2	1	2	pEG
3	2	2	1	pFG
4	1	1	1	pACB
4	0	2	2	pABD
4	2	2	0	pBCF
4	2	0	2	pACE
4	1	1	2	pABE, pACD
4	1	2	1	pABF, pBCD
4	2	1	1	pACF, pBCE

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4	1	2	2	pADF, pABG, pBDE
4	2	1	2	pACG, pCDE, pAEF
4	2	2	1	pBCG, pBEF, pCDF
4	2	2	2	pCDG, pAFG, pBEG, pDEF
4	1	1	3	pADE
4	1	3	1	pBDF
4	3	1	1	pCEF
4	1	2	3	pADG
4	3	2	1	pCFG
4	2	1	3	pAEG
4	2	3	1	pBFG
4	1	3	2	pBDG
4	3	1	2	pCEG
4	2	2	3	pDEG
4	3	2	2	pEFG
5	1	2	2	pABCD
5	2	1	2	pABCE
5	2	2	1	pABCF
5	2	2	2	pABCG, pABEF, pBCDE, pACDF
5	1	2	3	pABDE
5	2	1	3	pACDE
5	1	3	2	pABDF
5	2	1	3	pBCDF
5	3	1	2	pACEF
5	3	2	1	pBCEF
5	2	2	3	pABEG, pACDG, pADEF
5	2	3	2	pABFG, pBCDG, pBDEF
5	3	2	2	pACFG, pBCEG, pCDEF
5	1	3	3	pABDG
5	3	1	3	pACEG
5	3	3	1	pBCFG
5	2	3	3	pADFG, pBDEG
5	3	3	2	pBEFG, pDEFG
5	3	2	3	pCDEG, pAEFG
5	2	2	4	pADEG
5	2	4	2	pBDFG
5	4	2	2	pCEFG
5	3	3	3	pDEFG
6	2	2	3	pABCDE
6	2	3	2	pABCDF
6	3	2	2	pABCEF
6	2	3	3	pABCDG, pABDEF
6	3	2	3	pABCEG, pACDEF

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6	3	3	2	pABCFG, pBCDEF
6	4	3	2	pBCEFG
6	4	2	3	pACEFG
6	3	3	3	pABEFG, pACDFG, pBCDEG
6	3	4	2	pBCDFG
6	2	4	3	pABDFG
6	3	2	4	pACDEG
6	2	3	4	pABDEG
6	4	3	3	pCDEFG
6	3	4	3	pBDEFG
6	3	3	4	pADEFG
7	4	4	3	pBCDEFG
7	4	3	4	pACDEFG
7	3	4	4	pABDEFG
7	4	3	3	pABCEFG
7	3	4	3	pABCDFG
7	3	3	4	pABCDEG
7	3	3	3	pABCDEF
8	4	4	4	pABCDEFG

[0083] The decoding matrix provided in Table 4 is constructed using two assumptions. First, the decoding matrix assumes that the positive control (p) always produces a positive outcome. Second, the decoding matrix assumes that, within each color, the intensity is additive and scales in the same way with changing probe concentration, regardless of which probe the signal may come from. This essentially means that the signals are additive and digital. The conditions underlying these assumptions may be met by properly preparing the assay. If a fluorescent signal is used, the intensity need only be approximately additive and digital, as demonstrated in the Examples provided herein.

[0084] Table 4 allows the conversion of a cumulative measurement of intensity in four ranges of fluorescent wavelengths (i.e., signal intensity within each color range), into the corresponding analytes present in the sample. For example, a result of 4321 indicates that p, C, F, and G are present and the other analytes are not present. This result is referred to as a "legitimate" result, because it is present in the decoding matrix. By contrast, a result of 4000, while possible to measure, does not occur in the decoding matrix. More specifically, the result of 4000 cannot be achieved by adding any combination of control and analyte codes from Table 3. This result is

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referred to as an "illegitimate" result. An illegitimate result may indicate that the assay malfunctioned. Thus, the control (p) and the decoding matrix provide a means of verifying that the assay is functioning properly.

[0085] Table 4 is exhaustive for any combination of fluorophores generating four resolvable emission spectra (e.g., colors). The term "rank" is used to describe the number of detected analytes, including the control. In the example provided above, if the assay functions properly, the rank is equal to the value of the blue signal. For example, a rank of 8 indicates that the control and all seven other analytes (A-G) are present. A rank of 2 indicates that the control and only one analyte are present. The lowest rank is a rank of 1, which indicates that only the control is present. The number of possibilities at each rank can be enumerated. For example, continuing to refer to the encoding and decoding method described in Tables 3-4, there are 7 possibilities for rank 2. The number of possibilities at rank 3 can be calculated as a combination of 7 take 2, or 7!/(5! * 2!) = 21 possibilities. More generally, the number of possibilities for a combination of N take K is N!/((N-K)! * K!). Analogously, at ranks 4, 5, 6, 7, and 8, the number of possibilities is 35, 35, 21, 7, and 1, respectively. Referring to Table 4 shows that the table agrees with the theoretical prediction. Thus, Table 4 is an exhaustive decoding matrix for the encoding method provided in Table 3.

C. Reducing or Eliminating Degeneracy

[0086] The terms "degenerate" and "degeneracy," as used herein, generally describe a situation where a legitimate result is not definitive, because it can indicate more than one possibility in terms of the presence or absence of an analyte. For example, with reference to Table 4, result 5233 is degenerate because it can be decoded as either pADFG or pBDEG. Similarly, result 4222 is degenerate because it can be decoded as any of the following: pCDG, pAFG, pBEG, pDEF. By contrast, result 3110 can only indicate pBC and thus is not degenerate.

[0087] This disclosure provides methods of reducing or eliminating degeneracy, thereby increasing the confidence with which an analyte is detected. In one embodiment, degeneracy is eliminated by a method comprising (i) encoding each potential analyte to be detected as a value of a signal and, optionally, as a value in each of at least two components of a signal; (ii) enumerating every legitimate result that can be obtained from the coding scheme; (iii)

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identifying each legitimate result that is degenerate; and (iv) eliminating at least one potential analyte (or potential analyte code) from the coding scheme, wherein eliminating the at least one potential analyte reduces or eliminates degeneracy. For example, with reference to the coding scheme described in Table 3 and the decoding matrix described in Table 4 (enumerating every legitimate result), eliminating any two of analytes D, E, and F eliminates the degeneracy. Eliminating any one of analytes D, E, and F would not eliminate the degeneracy, but would reduce it.

[0088] With continued reference to the coding scheme described in Table 3, eliminating any two of analytes D, E, and F from the coding scheme results in a scheme where six analytes (including control) can be analyzed, with no degeneracy, using only 4 colors. By contrast, conventional methods of multiplexing would allow for only the reporting of 3 analytes and 1 control using 4 colors. Therefore, the number of analytes that can be analyzed is nearly doubled by using the methods provided herein.

[0089] **Fig. 2** shows an exemplary embodiment of the invention in which 4 colors are used to detect five analytes and a control. The analytes are nucleic acids from dengue virus ("Dengue Fever"), *Mycobacterium tuberculosis* ("Tuberculosis"), human immunodeficiency virus (HIV) p17 (P17), *Plasmodium* ("Malaria"), and herpes simplex ("Herpes"). The "colors" in this example are fluorophores attached to oligonucleotide probes that also comprise a quencher. The oligonucleotide probes will generally be different for different analytes, whereas the color is the same. For example, all probes designated as Probe 1 have a blue color but generally will have a different oligonucleotide sequence. Of course, probes designated as Probe 1 could also have the same oligonucleotides sequence, if the complementary sequence was present in each of the analytes. The mechanism of detection with these probes is described elsewhere in this disclosure. Probe 1 (blue) hybridizes to all six analytes, including control. Probe 2 (green) hybridizes to analytes from dengue virus, *Mycobacterium*, and HIV p17. Probe 3 (yellow) hybridizes to analytes from dengue fever and *Plasmodium*. Probe 4 hybridizes to analytes from dengue virus, *Mycobacterium*, and herpes simplex. The coding scheme defined by these probes is illustrated in Table 5. With reference to Table 3, potential analytes D (1011) and F (1110) have been eliminated from the coding scheme. Therefore, the coding scheme presented in Table

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5 is non-degenerate and each legitimate result from the assay corresponds to the presence or absence of a unique combination of analytes in a sample.

Table 5. Coding scheme for detection of dengue virus, *Mycobacterium*, HIV, *Plasmodium*, and herpes simplex, as exemplified in Fig. 2.

	Blue	**Green**	**Yellow**	**Red**
Ctrl. (p)	1	0	0	0
Herpes Simplex	1	0	0	1
Plasmodium	1	0	1	0
HIV	1	1	0	0
Mycobacterium	1	1	0	1
Dengue Virus	1	1	1	1

[0090] The methods for encoding and decoding presented above, including the methods for eliminating degeneracy, are all equally applicable to encoding methods using additional colors. For example, Table 3 could be extended by including additional colors and additional intensities (described further below). The decoding matrix is then generated as described above, enumerating every legitimate result that can be obtained from the coding scheme. A decoding matrix analogous to the decoding matrix provided in Table 4 may be constructed for any coding scheme described in this disclosure. The legitimate results that are degenerate are then identified and at least one potential analyte code is eliminated from the coding scheme to reduce or eliminate degeneracy. The method of eliminating degeneracy may be carried out using software on a computer readable medium, or hardware configured to carry out the method (e.g., a microchip).

[0091] Although degeneracy can be reduced or eliminated by the methods described above, and elsewhere in this disclosure, this disclosure also provides coding schemes that are non-degenerate by design. For example, after elimination of degeneracy in the coding scheme described in Table 3, the coding scheme may be extended indefinitely in a non-degenerate manner where the non-degeneracy is by design (see, e.g., the coding scheme exemplified in Table 6, described more fully below). Similarly, this disclosure provides coding schemes that

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are completely non-degenerate by design and therefore do not require any reduction or elimination of degeneracy (see, e.g., the coding scheme exemplified in Table 8, described more fully below). Thus, the coding schemes provided in this disclosure may have reduced or eliminated degeneracy, or be non-degenerate by design.

D. Encoding Methods Using More Than One Color and More Than One Intensity

[0092] The encoding method described above (e.g., Tables 3-5) may be further extended by allowing analytes to be encoded by an intensity greater than 1. For example, each of the analytes encoded in the coding scheme provided in Table 5 is encoded by a fluorescence intensity of either 1 or 0. Allowing higher values for the signal intensity in at least one color further increases the number of analytes that can be encoded by any of the methods provided in this disclosure. In some examples, these higher intensity values may be assigned any color except for the control color, in order to maintain the analyte counting capability of the control color.

[0093] Table 6 shows the first three tiers of an exemplary coding scheme that utilizes four colors and multiple intensities. Tier 1 of Table 6 is a reproduction of Table 3, showing the encoding of seven analytes and a control with four colors. As described above, any two of potential analytes D, E, and F may be eliminated from the coding scheme in order to produce a non-degenerate coding scheme. Tier 1 of Table 6 indicates that potential analytes D (1011) and F (1110) have been eliminated from the coding scheme to eliminate degeneracy. Therefore, the coding scheme presented in Tier 1 of Table 6 is capable of determining the presence of five analytes and one control using four colors, as described above.

[0094] The coding scheme of Tier 1 of Table 6 may be expanded to a second tier (Tier 2) by allowing the intensity in any of the colors to increase. As described above, the intensity of the control color may be maintained at 1, in order to preserve the sequence counting capability of the control. Increasing the intensity of any of the remaining three colors will yield codes 100Y, 10Y0, 1Y00, 10YY, 1Y0Y, 1YY0, and 1YYY, where $Y > 1$. The minimal value of Y for a new tier of encoding is equal to the cumulative maximum value from the prior tier(s) plus 1. As described below, a value greater than 1 could also be used, to maximize the differences between the intensities.

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[0095] Thus, in this context, the term "tier" is generally used to describe a set of codes that fully utilize the coding capability provided by a particular number of first values (e.g., intensities) and second values (e.g., colors), without degeneracy. For example, Tier 1 of Table 6 fully utilizes the coding capability provided by four colors with up to one intensity in each color, without degeneracy. As shown in Table 6, Tier 1, this results in six encoded analytes, including the control. To introduce a second tier, the minimum value of Y (described above) may be incremented to equal the cumulative maximum result from the prior tier(s) plus one (or more than one). In the example provided in Table 6, the intensity of the blue (control) color is maintained as one, to preserve the sequence counting capability in this color. Thus, Tier 2 consists of five non-degenerate encoding possibilities obtained by incrementing the intensities in the green, yellow, and red channels to equal the cumulative maximum results in each of these channels from Tier 1, plus one. All possibilities of these codes may then be enumerated for Tier 2, and codes resulting in degeneracy may be eliminated, or Tier 2 may be made non-degenerate by design, using the information used to eliminate the corresponding codes from Tier 1. Further coding capacity may then be achieved by adding a third tier, or further tiers, which are constructed according to analogous methods. A coding scheme may have an infinite number of tiers, for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100 or more tiers.

[0096] More specifically, with reference to Table 6, Tier 2, analyte H is encoded by 1004. The value of the control color is maintained as 1. The value of the red color is equal to the cumulative maximum result from the prior tier (3) plus 1, or 4. Similarly, analytes I and J are encoded by 1030 and 1400, respectively. Combinations of these codes are used to encode the remaining four analytes (K-N), as was done for analytes D-G in Tier 1. This completes Tier 2. In Tier 2, the inclusion of potential analytes K (1034) and M (1430) results in degeneracy. Therefore, these analytes have been eliminated from the coding scheme, to eliminate degeneracy.

[0097] Continuing to refer to Table 6, a third tier (Tier 3) is constructed using the same principles described above. Analyte O is encoded by 1-0-0-16. The value of the control color is still maintained as 1. The value of the red color is equal to the cumulative maximum result from the prior tiers (15) plus 1, or 16. Similarly, analytes P and Q are encoded by 1-0-9-0 and 1-16-0-

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0, respectively. Combinations of these codes are used to encode the remaining four analytes (R-U), as was done for analytes D-G in Tier 1 and analytes K-N in Tier 2. In Tier 3, the inclusion of potential analytes R (1-0-9-16) and T (1-16-9-0) results in degeneracy. Therefore, these analytes have been eliminated from the coding scheme, to eliminate degeneracy.

[0098] The three-tier coding scheme shown in Table 6 shows the encoding of 15 analytes and one control using four colors. This coding scheme may be indefinitely extended, by adding more intensities to generate additional tiers and/or adding more colors, to generate additional coding capability within the tiers. The methods of reducing or eliminating degeneracy, as described in this disclosure, may be used with the addition of each intensity and/or color, to reduce or eliminate degenerate results.

[0099] More generally, the coding scheme depicted in Table 6 is a non-degenerate, infinite extension of the coding scheme depicted in Tables 3-5. The maximum intensity of the cumulative measurement at the first tier is 6. The maximum intensity of the cumulative measurement at the second tier is 15. The maximum intensity of the cumulative measurement at the third tier is 63, and so on. Given a maximum cumulative intensity value (F), the maximum number of tiers (T) available in this coding scheme is $T = \log_4 (F + 1)$. The coding scheme depicted in Table 6 provides five non-degenerate codes per tier (P). Thus, the maximum number of codes $M = 5 * \log_4 (F + 1)$, or $M = P * T$, where P is the number of non-degenerate codes per tier and T is the number of tiers. For example, given $F = 63$, the maximum number of codes (i.e., analytes) is 15 for 5 non-degenerate codes per tier. This formula does not include the 1000 code, which is reserved for a positive control in Table 6. To include the control in the total number of analytes, one would simply add one, to provide the formula $M = (P * T) + 1$.

[00100] The methods provided in this disclosure may be used to expand this coding scheme infinitely. For example, by utilizing combinations of different intensities (i.e., first values) and colors (i.e., second values) one can encode any number of analytes (M) by varying the number of non-degenerate codes per tier (P) and the number of tiers (T). For example, the number of non-degenerate codes per tier is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more. Similarly, the number of tiers may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29,

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30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more.

Table 6. Encoding of 15 analytes and one control using four colors and multiple intensities.

Tier	Analyte	B	G	Y	R	Comments
1	**Ctrl. (p)**	1	0	0	0	
	A	1	0	0	1	
	B	1	0	1	0	
	C	1	1	0	0	
	~~**D**~~	~~1~~	~~0~~	~~1~~	~~1~~	Eliminated from coding scheme to eliminate degeneracy.
	E	1	1	0	1	
	~~**F**~~	~~1~~	~~1~~	~~1~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	G	1	1	1	1	
Cumulative Maximum Result		*6*	*3*	*2*	*3*	
2	**H**	1	0	0	4	
	I	1	0	3	0	
	J	1	4	0	0	
	~~**K**~~	~~1~~	~~0~~	~~3~~	~~4~~	Eliminated from coding scheme to eliminate degeneracy.
	L	1	4	0	4	
	~~**M**~~	~~1~~	~~4~~	~~3~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	N	1	4	3	4	
Cumulative Maximum Result		*11*	*15*	*8*	*15*	
3	**O**	1	0	0	16	
	P	1	0	9	0	
	Q	1	16	0	0	
	~~**R**~~	~~1~~	~~0~~	~~9~~	~~16~~	Eliminated from coding scheme to eliminate degeneracy.
	S	1	16	0	16	
	~~**T**~~	~~1~~	~~16~~	~~9~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	U	1	16	9	16	
Cumulative						

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Maximum Result						
Cumulative Maximum Result for Three Non-Degenerate Tiers		***16***	***63***	***26***	***63***	

[00101] The right-hand side of **Fig. 1** shows one exemplary embodiment of the method described above. More specifically, the right-hand side of **Fig. 1** shows an encoding method where nine or more analytes (Seq 1 - Seq 9, etc.) are each encoded by at least two colors, with varying intensities within each color. The color represents a fluorophore attached to an oligonucleotide probe that also comprises a quencher. The system can be designed so that each probe is labeled with single fluorophore, or each probe is labeled with more than one fluorophore. For example, the code for analyte H, in Table 6, is 1004. The intensity of 4 in the red channel may be achieved by either using an H-specific probe comprising 4 red fluorophores, or by using 4 H-specific probes each comprising a single red fluorophore. Of course, any combination of probes and fluorophores producing a result of 4 in the red channel would be equally appropriate, such as 2 probes with 2 red fluorophores each, and 1 probe with 1 red fluorophore and 1 probe with 3 red fluorophores, or simply one probe with one red fluorophore but present at 4x amount in the reaction mixture.

[00102] The coding scheme depicted on the right-hand side of **Fig. 1** may be represented as in Table 7. The result of the analysis shown on the right-hand side of **Fig. 1** is 4112, or an intensity of 4 in the blue channel, 1 in the green channel, 1 in the yellow channel, and 2 in the red channel. Using a decoding matrix constructed as described herein, this result is decoded to indicate the presence of Seq 1, Seq 3, Seq 4, and Seq 5.

Table 7. Coding scheme illustrated on the right-hand side of Fig. 1.

Tier	Analyte	B	G	Y	R	Comments
1	**Seq 1**	1	0	0	0	
	Seq 2	1	1	0	0	

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	Seq 3	1	0	1	0	
	Seq 4	1	0	0	1	
	Seq 5	1	1	0	1	
	Seq 6	1	1	1	1	
	~~**Eliminated Potential Seq**~~	~~1~~	~~0~~	~~1~~	~~1~~	Eliminated from coding scheme to eliminate degeneracy.
	~~**Eliminated Potential Seq**~~	~~1~~	~~1~~	~~1~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
Cumulative Maximum Result		*6*	*3*	*2*	*3*	
2	**Seq 7**	1	4	0	0	
	Seq 8	1	0	3	0	
	Seq 9	1	0	0	4	
Etc. - i.e. Continue as provided in Table 6.						

E. Encoding Methods Using One Color and One Intensity Per Analyte but Different Intensities Among Analytes

[00103] In some methods provided herein, each analyte is encoded by a single color and intensity combination. For example, in a four color system, the first four analytes may be encoded by 1000, 2000, 4000, and 8000. The next four analytes may be encoded by 0100, 0200, 0400, and 0800. Analytes 9-12 and 13-16 would be assigned analogously, as shown in Table 8.

[00104] Like the encoding method described in Table 6, this coding scheme is theoretically infinite, non-degenerate by construction, and only limited by the bandwidth of the instrument used to measure the signal. However, this coding scheme enables more analytes to be quantified per unit of bandwidth than the encoding method described in Table 6. The reason is that the available multiplicity of signal is used with maximal efficiency, as each level of intensity is utilized in the coding (i.e. there are no gaps in the chromatogram; see below for description of chromatograms). Table 8 shows one embodiment of this method, illustrating four tiers of encoding based on four colors and intensities 1, 2, 4, and 8. The coding scheme is non-degenerate, and the result if all 16 analytes are present is 15-15-15-15. This encoding method is more efficient, in terms of bandwidth utilization, than the encoding methods presented above.

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However, this method does not have the proofreading capability of the first scheme, as all the results decode to legitimate outcomes in the absence of gaps in the chromatogram.

Table 8. Example of encoding method using one color and one intensity per analyte, but different intensities among analytes.

Tier	Analyte	B	G	Y	R
1	**A**	1	0	0	0
	B	2	0	0	0
	C	4	0	0	0
	D	8	0	0	0
2	**E**	0	1	0	0
	F	0	2	0	0
	G	0	4	0	0
	H	0	8	0	0
3	**I**	0	0	1	0
	J	0	0	2	0
	K	0	0	4	0
	L	0	0	8	0
4	**M**	0	0	0	1
	N	0	0	0	2
	O	0	0	0	4
	P	0	0	0	8
		15	***15***	***15***	***15***

[00105] The method presented above, and exemplified in Table 8, may be extended by introducing additional colors and/or intensities. For example, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more colors may be used. Additional intensity levels, such as 16, 32, 64, 128, 255, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, and so on may also be used. The method is generalizable and may be used to encode an infinite number of analytes. Each analyte is represented by a code in a single color, wherein the value of

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the code in that single color equal to the sum of all previous values plus one. For example, if the first code contains a 1 in a particular color, the next codes are 2, 4, 8, 16, 32, 64, 128 and so on. Other progressions are possible, but will not be as efficient in terms of usage of the bandwidth. However, one of ordinary skill in the art will recognize that less efficient use of bandwidth may be desirable when one wishes to maximize separation between values. Similarly, the value 1 could be excluded from the coding scheme, for example to maximize the difference in the intensity between the first encoded value and instrumental noise. For example, in some cases a coding scheme may begin with a value of 2, which would provide a progression of 2, 4, 8, 16, 32, 64, 128, and so on for the analyte codes, while the progression of cumulative results would be 2, 4, 6, 8, 10, 12, 14, 16, and so on. This approach generates uniform gaps in the possible cumulative results, allowing for higher tolerance to noise in comparison to an analyte code progression of 1, 2, 4, 8, 16, 32, and so on, and its corresponding cumulative result progression of 1, 2, 3, 4, 5, 6, 7, 8, and so on. In this example, the increased robustness in the measurement comes at a cost of a decrease in the number of available codes within a fixed bandwidth of multiplicity. For example, if only 63 multiplicity states can be measured reliably, the coding scheme starting with 1 offers 7 codes per color whereas the coding scheme starting with 2 offers 6 codes per color. If 4 colors are available, the former will offer 28 codes, while the latter will offer 24 codes. In summary, both the starting signal intensity and the progression (i.e., difference between intensity values) may be scaled in order to maximize the ability to distinguish over instrumental noise and maximize the differences between the intensity values, thereby enhancing the ability to distinguish between distinct experimental outcomes.

[00106] The coding scheme illustrated in Table 8 is non-degenerate by design. Although the coding scheme illustrated in Table 8 uses both intensity and color to encode each of the 16 analytes, each of the analytes could also be encoded by simply utilizing intensity. For example, given the 16 analytes provided in Table 8 (A-P), a single color coding scheme encoding all 16 analytes may assign the values 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, and 16384 to analytes A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, and P, respectively. Such a coding scheme is non-degenerate by design and the cumulative intensity result can be unambiguously decoded to indicate the presence or absence of analytes A-P. This coding scheme is capable of detecting the presence or absence of M analytes, where $M = \log_2 (F + 1)$ and F is the maximum

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cumulative value of a signal (e.g., signal intensity). By adding a second component to this signal (e.g., color, as depicted in Table 8), the capacity of this coding scheme can be increased to $M = C * \log_2 (F + 1)$, where C is the number of colors used in said coding scheme.

[00107] As described throughout this specification, any suitable value may be used for C or F. For example, C may be at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more. F may be at least about 2, 4, 8, 16, 32, 64, 128, 255, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, 131072, 262144, 524288, 1048576, and so on. As described elsewhere in this disclosure, both the initial starting value of F and the steps in its progression may also be varied.

III. Analytes

[00108] An analyte may be any suitable analyte that may be analyzed using the methods and compositions of the invention, where the analyte is capable of interacting with a reagent in order to generate a signal with at least two components that can be measured. An analyte may be naturally-occurring or synthetic. An analyte may be present in a sample obtained using any methods known in the art. In some cases, a sample may be processed before analyzing it for an analyte. The methods and compositions presented in this disclosure may be used in solution phase assays, without the need for particles (such as beads) or a solid support.

[00109] In some cases, an analyte may be a polynucleotide, such as DNA, RNA, peptide nucleic acids, and any hybrid thereof, where the polynucleotide contains any combination of deoxyribo- and/or ribo-nucleotides. Polynucleotides may be single stranded or double stranded, or contain portions of both double stranded or single stranded sequence. Polynucleotides may contain any combination of nucleotides or bases, including, for example, uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine, isoguanine and any nucleotide derivative thereof. As used herein, the term "nucleotide" may include nucleotides and nucleosides, as well as nucleoside and nucleotide analogs, and modified nucleotides, including both synthetic and naturally occurring species. Polynucleotides may be any suitable polynucleotide for which one or more reagents (or probes) as described herein may be produced, including but not limited to cDNA, mitochondrial DNA (mtDNA), messenger RNA (mRNA),

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ribosomal RNA (rRNA), transfer RNA (tRNA), nuclear RNA (nRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small Cajal body-specific RNA (scaRNA), microRNA (miRNA), double stranded (dsRNA), ribozyme, riboswitch or viral RNA. Polynucleotides may be contained within any suitable vector, such as a plasmid, cosmid, fragment, chromosome, or genome.

[00110] Genomic DNA may be obtained from naturally occurring or genetically modified organisms or from artificially or synthetically created genomes. Analytes comprising genomic DNA may be obtained from any source and using any methods known in the art. For example, genomic DNA may be isolated with or without amplification. Amplification may include PCR amplification, multiple displacement amplification (MDA), rolling circle amplification and other amplification methods. Genomic DNA may also be obtained by cloning or recombinant methods, such as those involving plasmids and artificial chromosomes or other conventional methods (see Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*., cited *supra*.) Polynucleotides may be isolated using other methods known in the art, for example as disclosed in *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV) or *Molecular Cloning: A Laboratory Manual*. If the isolated polynucleotide is an mRNA, it may be reverse transcribed into cDNA using conventional techniques, as described in Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*., cited *supra*.

[00111] An analyte may be a protein, polypeptide, lipid, carbohydrate, sugar, small molecule, or any other suitable molecule that can be detected with the methods and compositions provided herein. An analyte may be an enzyme or other protein. An analyte may be a drug or metabolite (e.g., anti-cancer drug, chemotherapeutic drug, anti-viral drug, antibiotic drug, or biologic). An analyte may be any molecule, such as a co-factor, receptor, receptor ligand, hormone, cytokine, blood factor, antigen, steroid, or antibody.

[00112] An analyte may be any molecule from any pathogen, such as a virus, bacteria, parasite, fungus, or prion (e.g., PrP^{Sc}). Examples of viruses include those from the families Adenoviridae, Flaviviridae, Hepadnaviridae, Herpesviridae, Orthomyxoviridae, Papovaviridae, Paramyxoviridae, Picornaviridae, Polyomavirus, Retroviridae, Rhabdoviridae, and Togaviridae. Specific examples of viruses include adenovirus, astrovirus, bocavirus, BK virus, coxsackievirus, cytomegalovirus, dengue virus, Ebola virus, enterovirus, Epstein-Barr virus, feline leukemia

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virus, hepatitis virus, hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis D virus, hepatitis E virus, herpes simplex virus (HSV), HSV type 1, HSV type 2, human immunodeficiency virus (HIV), HIV type 1, HIV type 2, human papilloma virus (HPV), HPV type 1, HPV type 2, HPV type 3, HPV type 4, HPV type 6, HPV type 10, HPV type 11, HPV type 16, HPV type 18, HPV type 26, HPV type 27, HPV type 28, HPV type 29, HPV type 30, HPV type 31, HPV type 33, HPV type 34, HPV type 35, HPV type 39, HPV type 40, HPV type 41, HPV type 42, HPV type 43, HPV type 44, HPV type 45, HPV type 49, HPV type 51, HPV type 52, HPV type 54, HPV type 55, HPV type 56, HPV type 57, HPV type 58, HPV type 59, HPV type 68, HPV type 69, influenza virus, JC virus, Marburg virus, measles virus, mumps virus, Norwalk virus, parovirus, polio virus, rabies virus, respiratory syncytial virus, retrovirus, rhinovirus, rotavirus, Rubella virus, smallpox virus, vaccinia virus, West Nile virus, and yellow fever virus.

[00113] Examples of bacteria include those from the genuses *Bordetella, Borrelia, Brucella, Campylobacter*, *Chlamydia, Clostridium, Corynebacterium, Enterococcus, Escherichia, Francisella, Haemophilus, Helicobacter, Legionella, Leptospira, Listeria, Mycobacterium*, *Mycoplasma, Neisseria, Pseudomonas*, *Rickettsia, Salmonella*, *Shigella*, *Staphylococcus, Streptococcus*, *Treponema, Vibrio, and Yersinia.* Specific examples of bacteria include *Bordetella par apertussis, Bordetella pertussis, Borrelia burgdorferi, Brucella abortus, Brucella canis, Brucella melitensis, Brucella suis, Campylobacter jejuni, Chlamydia pneumoniae, Chlamydia psittaci, Chlamydia trachomatix, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium tetani, Corynebacterium diphtheriae, Enterococcus faecalis, Enterococcus faecium, Escherichia coli*, *Francisella tularensis, Haemophilus influenzae, Helicobacter pylori, Legionella pneumophila, Leptospira interrogans, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Mycobacterium ulcerans, Mycoplasma pneumoniae, Neisseria gonorrhoeae, Neisseria meningitidis, Pseudomonas aeruginosa, Rickettsia rickettsii, Salmonella choleraesuis, Salmonella dublin, Salmonella enteritidis, Salmonella typhi, Salmonella typhimurium, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus agalactiae, Streptococcus pneumoniae, Streptococcus pyogenes, Treponema pallidum, Vibrio cholerae, Yersinia pestis, and Yersinia enterocolitica.*

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[00114] Examples of parasites include those from the genuses *Acanthamoeba, Babesia, Balamuthia, Balantidium, Blasocystis, Cryptosporidium, Dientamoeba, Entamoeba, Giardia, Isospora, Leishmania, Naegleria, Pediculus, Plasmodium, Rhinosporidium, Sarcocystis, Schistosoma, Toxoplasma, Trichomonas,* and *Trypanosoma.* Specific examples of parasites include *Babesia divergens, Babesia bigemina, Babesia equi, Babesia microfti, Babesia duncani, Balamuthia mandrillaris, Balantidium coli, Dientamoeba fragilis, Entamoeba histolytica, Giardia lamblia, Isospora belli, Naegleria fowleri, Pediculus humanus, Plasmodium falciparum, Plasmodium knowlesi, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, Rhinosporidium seeberi, Sarcocystis bovihominis, Sarcocystis suihominis, Schistosoma mansoni, Toxoplasma gondii, Trichomonas vaginalis, Trypanosoma brucei,* and *Trypansoma cruzi.*

[00115] Examples of fungi include those from the genuses *Apophysomyces, Aspergillus, Blastomyces, Candida, Cladosporium, Coddidioides, Cryptococcos, Exserohilum, Fusarium, Histoplasma, Pichia, Pneumocystis, Saccharomyces, Sporothrix, Stachybotrys,* and *Trichophyton.* Specific examples of fungi include *Aspergillus fumigatus, Aspergillus flavus, Aspergillus clavatus, Blastomyces dermatitidis, Candida albicans, Coccidioides immitis, Crytptococcus neoformans, Exserohilum rostratum, Fusarium verticillioides, Histoplasma capsulatum, Pneumocystis jirovecii, Sporothrix schenckii, Stachybotrys chartarum,* and *Trichophyton mentagrophytes.*

[00116] In some cases, the methods provided in this disclosure may be used to detect any one of the analytes described above, or elsewhere in the specification. In some cases the methods provided in this disclosure may be used to detect panels of the analytes described above, or elsewhere in the specification. For example, a panel may comprise an analyte selected from the group consisting of any 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100 or more analytes described above or elsewhere in the specification.

[00117] An analyte may be obtained from any suitable location, including from organisms, whole cells, cell preparations and cell-free compositions from any organism, tissue, cell, or environment. Analytes may be obtained from environmental samples, biopsies, aspirates, formalin fixed embedded tissues, air, agricultural samples, soil samples, petroleum samples, water samples, or dust samples. In some instances, an analyte may be obtained from bodily

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fluids which may include blood, urine, feces, serum, lymph, saliva, mucosal secretions, perspiration, central nervous system fluid, vaginal fluid, or semen. Analytes may also be obtained from manufactured products, such as cosmetics, foods, personal care products, and the like. Analytes may be the products of experimental manipulation including, recombinant cloning, polynucleotide amplification, polymerase chain reaction (PCR) amplification, purification methods (such as purification of genomic DNA or RNA), and synthesis reactions.

[00118] More than one type of analyte may be detected in each multiplexed assay. For example, a polynucleotide, a protein, a polypeptide, a lipid, a carbohydrate, a sugar, a small molecule, or any other suitable molecule may be detected simultaneously in the same multiplexed assay with the use of suitable reagents. Any combination of analytes may be detected at the same time.

[00119] Detection of an analyte may be useful for any suitable application, including research, clinical, diagnostic, prognostic, forensic, and monitoring applications. Exemplary applications include detection of hereditary diseases, identification of genetic fingerprints, diagnosis of infectious diseases, cloning of genes, paternity testing, criminal identification, phylogeny, anti-bioterrorism, environmental surveillance, and DNA computing. For example, an analyte may be indicative of a disease or condition. An analyte may be used to make a treatment decision, or to assess the state of a disease. The presence of an analyte may indicate an infection with a particular pathogen, or any other disease, such as cancer, autoimmune disease, cardiorespiratory disease, liver disease, digestive disease, and so on. The methods provided herein may thus be used to make a diagnosis and to make a clinical decision based on that diagnosis. For example, a result that indicates the presence of a bacterial polynucleotide in a sample taken from a subject may lead to the treatment of the subject with an antibiotic.

[00120] In some cases the methods and compositions of the invention may be used to detect at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes. In some cases the methods and compositions of the invention may be used to detect 7-50, 8-40, 9-30, 10-20, 10-15, 8-12, or 7-12 analytes.

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[00121] In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis.

IV. Signals

[00122] The methods presented in this disclosure may be used with any quantifiable signal. In some cases, this disclosure provides coding schemes and methods to encode an infinite number of targets without degeneracy, using a single component of a signal (e.g., intensity). For example, as described above and in Example 3, a coding scheme may rely on a multiplicity of signal intensity without consideration of color. Although fluorescent probes have been used to illustrate this principle, the coding scheme is equally applicable to any other method providing a quantifiable signal, including an electrochemical signal and a chemiluminescent signal, as described elsewhere in this disclosure.

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[00123] The methods presented in this disclosure may also utilize the measurement of a signal in at least two dimensions, also referred to as the measurement of at least two components of a signal. In comparison to the coding scheme described in the paragraph above, which relies on, for example, signal intensity to differentiate between analytes, utilization of at least two components of a signal (e.g., color and intensity) allows the generation of more unique codes per unit of signal intensity bandwidth. When at least two components of a signal are utilized, a cumulative measurement of the at least two components may be obtained for a single sample volume. For example in the coding scheme described in Table 5, the presence of each analyte results in a particular intensity (one component of the signal) in each of the four colors (a second component of a signal). The combination of these constituent signals leads to a cumulative signal that may be measured by measuring an intensity at each wavelength or range of wavelengths. The corresponding coding scheme or decoding matrix may then be used to convert the cumulative measurement into a determination of the presence or absence of an analyte.

[00124] In some cases, a quantifiable signal comprises a waveform that has both a frequency (wavelength) and an amplitude (intensity). A signal may be an electromagnetic signal. An electromagnetic signal may be a sound, a radio signal, a microwave signal, an infrared signal, a visible light signal, an ultraviolet light signal, an x-ray signal, or a gamma-ray signal. In some cases, an electromagnetic signal may be a fluorescent signal, for example a fluorescence emission spectrum that may be characterized in terms of wavelength and intensity.

[00125] In certain portions of this disclosure, the signal is described and exemplified in terms of a fluorescent signal. This is not meant to be limiting, and one of ordinary skill in the art will readily recognize that the principles applicable to the measurement of a fluorescent signal are also applicable to other signals. For example, like fluorescent signals, any of the electromagnetic signals described above may also be characterized in terms of a wavelength and an intensity. The wavelength of a fluorescent signal may also be described in terms of color. The color may be determined based on measuring intensity at a particular wavelength or range of wavelengths, for example by determining a distribution of fluorescent intensity at different wavelengths and/or by utilizing a band pass filter to determine the fluorescence intensity within a particular range of wavelengths. Such band pass filters are commonly employed in a variety of

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laboratory instrumentation, including quantitative PCR machines. Intensity may be measured with a photodetector. A range of wavelengths may be referred to as a "channel."

[00126] In some cases, the methods provided in this disclosure may be used with any signal where the cumulative signal scales with the constituent signals of the same color, frequency, absorption band, and so on. However, the cumulative signal need not be digital or scale linearly with the number and intensity of the constituent signals. For example, if the physical principle of measurement is absorption, the cumulative attenuation is a product of constituent attenuations while the constituent concentrations are additive, due to the exponential nature of the Beer-Lambert law. The logarithm of the cumulative attenuation will then scale linearly with constituent concentrations in each absorption band (the equivalent of color, if fluorescent detection is used). The methods of the invention are therefore applicable. The methods of the invention may also be used with chemiluminescent signals and electrochemical signals.

[00127] The number of signals, and the number of dimensions or components measured, may also be expanded beyond the numbers shown in the exemplary embodiments of the invention, leading to an expansion in multiplexing capability. The exemplary embodiments provided in this disclosure utilize coding schemes constructed utilizing a fluorescent signal with one or two components measured: wavelength and/or intensity. The number of analytes that can be encoded can be increased by increasing the number of wavelengths and/or intensities. The number of analytes that can be encoded can also be increased by increasing the number of signals, for example by combining a fluorescent signal with an electrochemical signal or a FRET signal (fluorescence resonance energy transfer).

[00128] In some cases, more than two components of a signal may be measured. For example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, 20 or more components of a signal may be measured. At least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, or 20 components of a signal may be measured. At least 2, but fewer than 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, or 20 components of a signal may be measured. In some cases, 2-3, 2-4, 2-5, 2-6, 3-5, 3-6, 3-8, or 5-10 components of a signal may be measured. These additional components may include kinetic components, such as a rate of signal decay and rate of photobleaching.

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[00129] If a fluorescent signal is employed, the number of analytes that can be encoded may be further expanded by utilizing additional fluorophores. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores may be used. In some cases, fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores may be used. In some cases, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 fluorophores may be used.

[00130] Generally, the number of analytes that can be encoded may be further expanded by utilizing additional first values (e.g., intensities) that are values or ranges of values from a first component of a signal. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more first values that are values or ranges of values from a first component of a signal may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more first values that are values or ranges of values from a first component of a signal may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 first values that are values or ranges of values from a first component of a signal may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 first values that are values or ranges of values from a first component of a signal may be used.

[00131] In instances in which intensity is a component of a signal that is quantified, such as when a fluorescent signal is utilized, the presence of an analyte may be encoded using a variety of intensities or ranges of intensities. For example, a coding scheme may utilize 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more intensities or ranges of intensities. A coding scheme may utilize at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,

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14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more intensities or ranges of intensities. A coding scheme may utilize fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 intensities or ranges of intensities. In some cases, a coding scheme may utilize 2-20, 2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9, 2-10, 3-4, 3-5, 3-6, 3-7, 3-8, 3-9, 3-10, 4-5, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, or 5-10 intensities or ranges of intensities.

[00132] The number of analytes that can be encoded may be further expanded by utilizing additional second values (e.g., wavelengths) that are values or ranges of values from a second component of a signal. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second values that are values or ranges of values from a second component of a signal may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more second values that are values or ranges of values from a second component of a signal may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 second values that are values or ranges of values from a second component of a signal may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 second values that are values or ranges of values from a second component of a signal may be used.

[00133] In instances in which wavelength is a component of a signal that is quantified, such as when a fluorescent signal is utilized, the presence of an analyte may be encoded using a variety of wavelengths or ranges of wavelengths. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more wavelengths or ranges of wavelengths may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more wavelengths or ranges of wavelengths may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27,

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28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 wavelengths or ranges of wavelengths may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 wavelengths or ranges of wavelengths may be used.

[00134] In some cases, when degeneracy is eliminated, the methods of the invention are capable of detecting the presence or absence of at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more analytes in a single volume when each reagent used to generate a signal in the volume generates only one second value (e.g., each reagent, emits light at only one wavelength).

[00135] In other cases, when degeneracy is eliminated, the methods of the invention are capable of detecting the presence or absence of at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more analytes in a single volume using a total of four second values in that volume (e.g., a total of four wavelengths or ranges or wavelengths, which might be implemented by using four spectrally resolvable fluorophores).

[00136] As described throughout the specification, the assay provided herein utilizes cumulative measurements on a sample. A cumulative measurement may be, for example, a single measurement of intensity values, or a measurement of intensity values at one or more wavelengths or ranges of wavelengths. A plurality of cumulative measurements may be obtained. For example, an intensity may be measured at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more wavelengths or ranges of wavelengths. An intensity may be measured at at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more wavelengths or ranges of wavelengths. An intensity may be measured at less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 wavelengths or ranges of wavelengths.

[00137] More generally a cumulative measurement may be obtained for any quantifiable component of a signal, and for any quantifiable component of a signal at another quantifiable

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component of a signal. For example, at least a first component of a signal may be measured at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second components of a signal. At lest a first component of a signal may be measured at at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second components of a signal. At least a first component of a signal may be measured at less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 second components of a signal.

[00138] As is apparent from this disclosure, each analyte to be detected can be encoded as a code utilizing any number of suitable components of a signal or any number of signals. For example, each analyte to be detected can be encoded in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more components of a signal or signals. In some cases, each analyte to be detected can be encoded in at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more components of a signal or signals. In some cases, each analyte to be detected can be encoded in fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 components of a signal or signals. In some cases, 1-4, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 components of signal or signals may be used to encode each analyte. Each analyte in a coding scheme may be encoded by the same number of components of a signal, or signals, or different numbers of components of a signal, or signals.

A. Probes, Primers, Fluorophores, and Quenchers

[00139] Some of the methods provided in this disclosure utilize a reagent that generates a signal in the presence of an analyte. Any suitable reagent may be used with the invention. Generally, a reagent will have an analyte-specific component and a component that generates a

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signal in the presence of the analyte. In some cases, these reagents are referred to as probes. The probes may be hybridization probes. The hybridization probes may be an oligonucleotide probe attached to a fluorophore and a quencher (e.g., a TAQMAN probe).

[00140] The methods of the invention may use one or more reagents or probes to detect the presence or absence of each analyte. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more or probes may be used to detect the presence or absence of each analyte. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 probes may be used to detect the presence or absence of each analyte. In some cases, fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, , 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 probes may be used to detect the presence or absence of each analyte. In some cases, the number of probes used to detect the presence or absence of each analyte is 1-10, 2-9, 2-8, 2-7, 2-6, 2-5, 2-4, 1-3, or 1-4.

[00141] In some cases, a sample is contacted with 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more probes to detect the presence or absence of all analytes. In some cases, a sample is contacted with at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more probes to detect the presence or absence of all analytes. In some cases, a sample is contacted with fewer than 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 probes to detect the presence or absence of all analytes. In some cases, the number of probes that a sample is contacted with to detect the presence or absence of all analytes is 4-50, 4-40, 4-30, 4-20, 5-15, 5-10, or 3-10.

[00142] As described above, oligonucleotide probes attached to a fluorophore and a quencher may be used to detect the presence of an analyte in a polynucleotide amplification assay. So long as the quencher and the fluorophore are in proximity, the quencher quenches the fluorescence emitted by the fluorophore upon excitation by a light source. The sequence of the

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oligonucleotide probe is designed to be complementary to a polynucleotide sequence present in an analyte, and therefore capable of hybridizing to the polynucleotide sequence present in the analyte. Hybridization of the oligonucleotide probe may be performed in a nucleic acid amplification reaction comprising primers (e.g., a polymerase chain reaction). Upon extension of the primers by a DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and the quencher into the medium. The proximity between the fluorophore and the quencher is broken and the signal from the fluorophore is no longer quenched. Thus, the amount of fluorescence detected is a function of the amount of analyte present. If no analyte is present, the probe will not hybridize to an analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be produced.

[00143] Oligonucleotide probes may have one or a plurality of fluorophores and quenchers per probe. For example, in some embodiments an oligonucleotide probe may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores. An oligonucleotide probe may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores. An oligonucleotide probe may comprise fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores.

[00144] An oligonucleotide probe may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more quenchers. An oligonucleotide probe may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 quenchers. An oligonucleotide probe may comprise fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 quenchers.

[00145] Attachment of probes and quenchers to a probe may be performed in the same reaction or in serial reactions. A series of reactions may be performed to label probes with at

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least one fluorophore and the reaction products may be mixed to generate a mixture of probes with different fluorophores.

[00146] If an oligonucleotide probe comprises two or more fluorophores, these fluorophores may be arranged to allow Förster (Fluorescence) resonance energy transfer (FRET) to occur between the fluorophores. Briefly, FRET is a mechanism of energy transfer between fluorophores. Using FRET-based probes allows one excitation source to generate two different fluorescence emission signals by excitation of a single fluorophore. For example, the methods described herein may used paired probes, where one probe in the pair is attached to a fluorophore and a quencher and a second probe is attached to two fluorophores (in close enough proximity for FRET to occur) and a quencher. Any combination of fluorophores and quenchers that provide for FRET may be used. Such an approach doubles the number of fluorescent probes that may be used with an excitation source, because a single excitation source can be used to produce two signals: one from the probe with one fluorophore and one from the probe with two fluorophores in close enough proximity to provide for FRET. For example, using the encoding method described in Table 8, the number of unambiguously detectable analytes could be increased from 16 to 32 by pairing each of the probes in Table 8 with a corresponding FRET probe.

[00147] The primers may be specific for a particular analyte and capable of amplifying a region complementary to a probe. In some cases, the number of pairs of primers used is equivalent to the number of probes. In other cases, the number of probes used may exceed the number of primer pairs used. In still other cases, the number of primer pairs used may exceed the number of probes used. In some cases a sample to be analyzed is contacted with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more pairs of primers. In some cases a sample to be analyzed is contacted with at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more pairs of primers. In some cases a sample to be analyzed is contacted with fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44,

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45, 46, 47, 48, 49, or 50 pairs of primers. In some cases, the number of pairs of primers is 2-10, 3-15, 4-20, 3-10, 4-10, 5-10, 6-8, or 6-10.

[00148] The primers may amplify regions of a polynucleotide in which different numbers of hybridization probes hybridize. For example, at least one pair of primers may amplify a region complementary to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 hybridization probes. In some cases, all of said pairs of primers may amplify a region complementary to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 hybridization probes.

[00149] In one example, a first probe may be labeled with the fluorophore 5'-fluorescein amidite (5'-FAM) and the quencher black hole quencher 1 (BHQ-1). A second probe may be labeled with 5'-FAM in close proximity (e.g., attached to) to cyanine 5.5 (Cy5.5) and the quencher black hole quencher 3 (BHQ-3). Upon digestion of the probe, via the nuclease activity of the polymerase, two fluorescent signals are generated from a single excitation wavelength (e.g., 470 nm). The fluorophore from the first probe will fluoresce at about 520 nm. The fluorophores on the second probe undergo FRET. The donor fluorophore, FAM is excited by the excitation light source (e.g., 470 nm) and transfers its energy to the acceptor fluorophore, Cy5.5. The acceptor fluorophore emits at about 705 nm. These fluorophores and quenchers are merely exemplary. Any fluorophores that can undergo FRET and any quenchers that can quench fluorescence are suitable for use with the invention. Methods for producing FRET-based probes are described in Jothikumar et al., BioTechniques, 2009, 46(7):519-524.

[00150] In some examples, a single hybridization probe may be used for each analyte. In order to utilize multi-color encoding (e.g., as shown in Table 3), each probe may be labeled with a plurality of fluorophores at pre-determined ratios. The ratio may be determined so that a positive control signal provides the same intensity as other positive control signals of the same intensity, in the same color. This approach reduces the number of probes that must be synthesized and may be less expensive to deploy than methods utilizing multiple probes. Moreover, in the case of hybridization probes, it is easier to fit one hybridization probe to an analyte sequence than multiple probes.

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[00151] Although many aspects of the invention are exemplified using nucleic acid-based probes, one of ordinary skill in the art will readily recognize that other forms of probes would work equally well with the invention described in this disclosure. For example, a binding molecule specific to an analyte could be used as a probe. Non-limiting exemplary binding molecules include an antibody recognizing an analyte, and generating a signal in the presence of an analyte.

[00152] In embodiments of the invention that utilize fluorescent labels, any suitable fluorescent label may be used. Exemplary fluorescent labels suitable for use with the invention include rhodamine, rhodol, fluorescein, thiofluorescein, aminofluorescein, carboxyfluorescein, chlorofluorescein, methylfluorescein, sulfofluorescein, aminorhodol, carboxyrhodol, chlororhodol, methylrhodol, sulforhodol; aminorhodamine, carboxyrhodamine, chlororhodamine, methylrhodamine, sulforhodamine, and thiorhodamine, cyanine, indocarbocyanine, oxacarbocyanine, thiacarbocyanine, merocyanine, cyanine 2, cyanine 3, cyanine 3.5, cyanine 5, cyanine 5.5, cyanine 7, oxadiazole derivatives, pyridyloxazole, nitrobenzoxadiazole, benzoxadiazole, pyrene derivatives, cascade blue, oxazine derivatives, Nile red, Nile blue, cresyl violet, oxazine 170, acridine derivatives, proflavin, acridine orange, acridine yellow, arylmethine derivatives, auramine, crystal violet, malachite green, tetrapyrrole derivatives, porphin, phtalocyanine, and bilirubin. Exemplary quenchers include black hole quenchers, such as BHQ-0, BHQ-1, BHQ-2, BHQ-3; ATTO quenchers, such as ATTO 540Q, ATTO580Q, and ATTO612Q; and the like.

[00153] The fluorophores that may be used with the invention are not limited to any of the fluorophores described herein. For example, fluorophores with improved properties are continually developed, and these fluorophores could readily be used with the methods provided in this disclosure. Such improved fluorophores include quantum dots, which may emit energy at different wavelengths after being excited at a single wavelength. The advantage of using such fluorophores is that only a single excitation source is needed, but many different signals may be quantified, for example in terms of color and intensity. Moreover, fluorophores with narrow emission spectra would be particularly useful with the method described herein, as such fluorophores could be included in multiplex assays with minimal or no overlap between their

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emission spectra, thereby offering many more "colors" and boosting significantly the overall number of coded analytes.

[00154] In some cases, one or more reagents are lyophilized. Any suitable reagent may be lyophilized. For example, probes, primers, enzymes, antibodies, or any other reagent used for detection may be lyophilized. In some cases, a sample comprising an analyte may also be lyophilized. Lyophilization may be useful, for example, when distributing reagents and/or samples in developing regions where access to cold storage is expensive, not readily available, or not reliably available. In one example, lyophilized reagents comprise any probe or analyte-specific reagent described in this disclosure or otherwise suitable for use with the invention. In another example, lyophilized reagents comprise PCR primers. In yet another example, lyophilized reagents comprise reagents suitable for performing a PCR reaction.

V. Analytical Techniques and Instrumentation

[00155] The methods provided herein are suitable for use with a variety of detection methods. For example, the methods may be applied using an analytical technique that measures the wavelength and intensity of a fluorescent signal. This may be accomplished by measuring the intensity of a signal across a spectrum of wavelengths, or by using band pass filters that restrict the passage of certain wavelengths of light, thereby allowing only light of certain wavelengths to reach a photodetector. Many real-time PCR and quantitative PCR instruments comprise an excitation light source and band pass filters that enable the detection of fluorescent signals in four colors (e.g., blue, green, yellow, and red). Therefore, the methods of the invention can be readily applied using instruments widely used in the art. Importantly, the methods and compositions provided herein may be used to detect multiple analytes by obtaining a cumulative measurement on a single solution. No separation is necessary. The invention does not require the use of beads or a solid phase. Of course, one of ordinary skill in the art would understand that the invention could be used with separation, beads, or a solid phase, if desired.

[00156] **Fig. 5** shows two examples of instrumental configurations that may be used to obtain cumulative measurements useful for the methods of the invention. With reference to **Fig. 5A**, an instrument with a detector configured to detect both wavelength and intensity (e.g., a fluorometer) is shown ~~in 501.~~ With reference to ~~501~~**Fig. 5A**, at least one excitation light source

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502 is directed into a chamber **503** containing analytes and reagents that generate a signal in the presence of the analytes **504.** As described elsewhere, the analyte may be a nucleic acid and the reagents generating the signal may be hybridization probes comprising a fluorophore and a quencher. If the analyte is present, an emission signal **505** is generated. In the configuration shown in ~~501~~**Fig. 5A**, the wavelength and intensity of this emission signal are measured across a spectrum by a detector capable of generating a fluorescence emission spectrum **506.**

[00157] The wavelength and intensity may also be determined using a combination of a photodetector and band pass filters. This configuration is used in several thermal cyclers known in the art. With reference to **Fig. 5B**, an instrument with band pass filters and a photodetector is depicted ~~in 507.~~ With reference to ~~507~~**Fig. 5B**, at least one excitation light source **508** is directed into a chamber **509** containing analytes and reagents that generate a signal in the presence of the analytes **510.** As described elsewhere, the analyte may be a nucleic acid and the reagents generating the signal may be hybridization probes comprising a fluorophore and a quencher. If the analyte is present, an emission signal **511** is generated. In the configuration shown in ~~507~~**Fig. 5B**, band pass filters **512, 513,** and **514** are used to restrict the passage of light to light within certain ranges of wavelengths. For example band pass filter **512** may restrict the passage of light to light within wavelength range 1. Band pass filter **513** may restrict the passage of light to light within wavelength range 2. Band pass filter **514** may restrict the passage of light to light within wavelength range 3. Any number of band pass filters (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more) may be used with the invention. In addition, the positions of the band pass filters in ~~507~~ **Fig. 5B** are not meant to be limiting. In some cases, emitted light may be passed through more than one band pass filter at any time. In other cases, the band pass filters may be configured so that all emitted light passes through only one band pass filter at a time. After passage through a band pass filter, filtered light **515, 516,** and **517** is detected by a photodetector. For example, filtered light **515** may be light within wavelength range 1, filtered light **516** may be light within wavelength range 2, and filtered light **517** may be light within wavelength range 3. The intensity of each of the filtered lights **515, 516,** and **517** may then be quantified by a photodetector **518.**

[00158] The methods described in this disclosure are compatible with a variety of amplification methods, including quantitative PCR (qPCR) methods, end point PCR methods,

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reverse transcriptase PCR, and digital PCR methods. Digital PCR methods (e.g., DROPLET DIGITAL (BIORAD) and DYNAMIC ARRAY (FLUIDIGM)) produce highly sensitive quantification of polynucleotide copy numbers. The methods provided herein can be easily integrated into these systems to significantly expand their throughput by allowing multiplexing in a droplet or a dynamic array.

[00159] Similarly, the methods described in this disclosure may be applied in a real-time PCR assay. For example, real-time data may be fully recorded as the PCR runs to completion. The end-point values may then be decoded to indicate the presence or absence of analytes. The individual cycle thresholds (Ct) values may be analyzed for each color by detecting the maxima of the second derivative of the real-time curve in that color. As a particular analyte is amplified, fluorophores are released from its corresponding probes in the same ratios as the color multiplicities in the coding of that sequence. Hence, e.g. a code of 1100 will have the same Ct in blue and green, while a code of 1400 will have its green Ct precede its blue Ct by 2 cycles. This additional data enables determination of the identity and starting quantity of each analyte.

[00160] The methods described herein may also be used directly on a tissue sample. For example, a tissue sample may be obtained and photolithographic methods may be used to build wells directly onto the tissue sample. The tissue sample may be fixed prior to building the wells. The tissue sample may then be analyzed by dispensing appropriate reagents into the wells in the tissue sample. The encoding and decoding methods described in this disclosure may be used for the multiplexed detection of analytes within the wells etched into the tissue. Each well may correspond to a single cell or a few cells, providing excellent spatial resolution when analyzing different portions of a tissue for an analyte. This method may be used to detect analytes in different areas of the same tissue.

[00161] In some cases, instruments may be modified or constructed, for example, to provide additional excitation light sources, at multiple wavelength, and/or to provide additional band pass filters or a capability of determining a complete spectrum. Including additional excitation sources would allow for the excitation of a larger variety of fluorophores. Including additional band pass filters, or modifying or constructing an instrument capable of determining an entire spectrum allows detection of a wider variety of emissions from fluorophores. These techniques can be used to increase the number of fluorophores that can be used with the methods

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described in this disclosure and, accordingly, to increase the number of analytes that be simultaneously detected.

[00162] In some cases, the methods described in this disclosure utilize end-point PCR methods. However, in some cases a result may be obtained before the end-point. This may be advantageous when, for example, results are needed as soon as possible. For example, in one case calibration experiments are performed in which different starting concentrations of an analyte are analyzed to determine the cycle number at which the signal becomes saturated, as a function of the starting amount of the analyte. Using this data, a computer monitoring a PCR reaction in real-time can be programmed to search for saturation up to the maximal cycle number according to the limit of detection (LOD) of the particular system. If there is no amplification of an analyte (e.g., other than a positive control, if present), by the maximal cycle number, the result for that analyte is negative. If there is amplification of the analyte by the maximal cycle number, the result for that analyte is positive and the saturation intensity in each color may be used to decode the result using the coding scheme. In both cases, there would be no need to run the PCR reaction beyond the number of cycles set by the LOD for that instrument. Although PCR has been used to illustrate this quantitative method, one of ordinary skill in the art will readily recognize that similar principles could be applied in any catalytic system where the starting amount of an analyte limits the rate of reaction, and the rate of reaction can be measured and reported by an evolution in a signal intensity over time.

VI. Compositions and Kits

[00163] This disclosure also provides compositions and kits for use with the methods described herein. The compositions may comprise any component, reaction mixture and/or intermediate described herein, as well as any combination. For example, the disclosure provides detection reagents for use with the methods provided herein. Any suitable detection reagents may be provided, including hybridization probes labeled with a fluorophore and a quencher and primers, as described elsewhere in the specification.

[00164] In some cases, compositions comprise reagents for the detection of at least seven analytes using four fluorophores. In some cases, compositions comprise reagents for the detection of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26,

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27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using four fluorophores. In some cases, compositions comprise reagents for the detection of at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using five fluorophores. In some cases, compositions comprise reagents for the detection of at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using six fluorophores. In some cases, compositions comprise reagents for the detection of at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using seven fluorophores.

[00165] In some cases the compositions comprise primers. The compositions may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 pairs of primers.

[00166] The invention also provides kits for carrying out the methods of the invention. Accordingly, a variety of kits are provided in suitable packaging. The kits may be used for any one or more of the uses described herein, and, accordingly, may contain instructions for detecting the presence or absence of each analyte of a plurality of analytes. A kit may comprise a coding scheme, or a decoding matrix, to assist the user in converting a cumulative measurement to a result indicating the presence or absence of each of a plurality of analytes. A kit may be a diagnostic kit, for example, a diagnostic kit suitable for the detection of any analyte, including the analytes recited herein. A kit may contain any of the compositions provided in this disclosure, including those recited above.

[00167] **Fig. 6** shows schematics of exemplary kits of the invention. With reference to **Fig. 6**, a kit comprising a kit body **601** is shown that contains reagents for the detection of analytes (e.g., polynucleotide probes comprising a fluorophore and a quencher) **602** and primers for the amplification of a nucleic acid **603.** The kit may also contain any other reagents, such as reagents suitable for performing an amplification reaction **604.** In the embodiment depicted in **601**, the reagents for the detection of analytes and the primers are each contained in a single volume (e.g., tube or a bottle). However, these reagents may also be provided in separate

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volumes, as depicted the kit body shown in **605.** The kit body **605** contains seven separate volumes. Three volumes (**606, 607,** and **608**) contain reagents for the detection of analytes (or mixtures of such reagents) and three volumes (**609, 610,** and **611**) contain primers (or mixtures of primers). The kit may also contain reagents suitable for performing an amplification reaction **604.**

[00168] **Fig. 6** is provided for illustrative purposes only. Any number of reagents for the detection of analytes (e.g., probes) and any number of primers may be included in a single tube or bottle, as appropriate for the application. For example, in some embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more reagents for the detection of analytes may be included in a single bottle. In other cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more primers for amplification may be included in a single bottle or tube. In some cases, primers and probes may be provided in the same bottle or tube. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more probes may be provided per pair of primers. In other cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more primers may be provided per pair of probes. The kits may contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more bottles or tubes. Control analytes, and their associated detection reagents and primers, may also be included in the kit. These may be included in a separate bottle or tube or, for example, included within the bottle or tube providing reagents for an amplification reaction **604**. As described elsewhere in the specification, any reagent in the kits may be lyophilized.

VII. Systems and Software

[00169] The methods of this disclosure may also be performed as part of a system. For example, one or more steps of the methods described herein may be performed by software contained on a computer readable medium. The software may be used to encode analytes, check

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for degeneracy, reduce or eliminate degeneracy, and/or produce a decoding matrix. The software may also be used to convert cumulative measurements made on an instrument (e.g., a PCR machine) into information concerning the presence or absence of an analyte. For example, with reference to **Fig. 4**, a computer **401** may be connected to an input device **403**, a display **402**, and a printer **404**. The computer may execute one or more of the steps of any of the methods described in this disclosure, including, for example, encoding methods **410**, analysis methods **420**, and decoding methods **430.** A computer may reduce or eliminate degeneracy, for example as part of the encoding methods **410.** Similarly, a computer may generate a coding schme that is non-degenerate by design using, for example, mathematical iteration. A computer may also be connected to a network **405**. The network may be a local network and/or the Internet. The network may be used to perform the methods of the invention on multiple computers in different locations. For example, one computer may perform the encoding, another computer may perform the measuring, and yet another computer may perform the decoding. A computer may perform any or all of these methods. The network may be used to transmit information obtained using the methods described in this disclosure throughout the world, for example across a border.

[00170] In one example, the software is embedded in the instrument making the measurement, such as a thermal cycler. In another example, the software is installed on a computer attached to the instrument making the measurement, such as a computer attached to a thermal cycler. In another example, the software is in the "cloud" -- i.e., on a computer that another computer may communicate with through a network.

[00171] Each of the elements described above may be connected to a controller that communicates with each of the elements and coordinates their actions. For example, a controller may initiate the encoding of a series of analytes into values of color and intensity. As described throughout this disclosure, the controller can execute software to reduce or eliminate degeneracy by removing one or more potential analyte codes from the encoding matrix. The controller may then automatically order probes corresponding to this code from a manufacturer of probes. Alternatively, the controller may operate an oligonucleotide synthesis instrument that can synthesize the appropriate probes. After analysis of the sample on the instrument (which may also be automated), the controller may process the results of the analysis using the decoding matrix. The controller may then provide these results to a user.

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VIII. Services

[00172] The methods provided herein may also be performed as a service. For example, a service provider may obtain the identity of a plurality of analytes that a customer wishes to analyze. The service provider may then encode each analyte to be detected by any of the methods described herein and provide appropriate reagents to the customer for the assay. The customer may perform the assay and provide the results to the service provider for decoding. The service provider may then provide the decoded results to the customer. The customer may also encode analytes, generate probes, and/or decode results by interacting with software installed locally (at the customer's location) or remotely (e.g., on a server reachable through a network). Exemplary customers include clinical laboratories, physicians, manufacturers of food and consumer products, industrial manufacturers (e.g., petroleum companies) and the like. A customer or party may be any suitable customer or party with a need or desire to use the methods, systems, compositions, and kits of the invention.

EXAMPLES

Example 1: General Materials & Methods

1. General Reagents

[00173] TE Buffer, pH7 (LIFE TECHNOLOGIES, Carlsbad, CA); UltraPure RNAse-free Water (LIFE TECHNOLOGIES, Carlsbad, CA); Taq 5x Master Mix (FISHER SCIENTIFIC COMPANY, Tustin, CA).

2. DNA Sequences, Primers, and Probes

[00174] Five nucleic acids from organisms of clinical relevance were chosen for the exemplary study: (1) human immunodeficiency virus 1 (HIV-1); (2) *Plasmodium falciparum* (Malaria); (3) herpes simplex virus-2 (HSV-2); (4) *Mycobacterium tuberculosis* (TB); (5) and dengue virus type 3 (dengue fever).

[00175] Two regions of diagnostic relevance from the HIV-1 genome, p17 and poly-protease, were selected from the Los Alamos National Laboratory HIV-1 reference sequence. A diagnostic sequence from the *Plasmodium falciparum* ChR7 gene was obtained from the UCSC *Plasmodium falciparum* Genome Browser. A sequence for Herpes Simplex Virus-2 was synthesized from the sequence obtained from the European Molecular Biology Library.

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Similarly a diagnostic sequence for the rpoB gene in *Mycobacterium tuberculosis* was synthesized from a sequence obtained from the European Molecular Biology Library. A PCR diagnostic sequence for Dengue Virus Type 3 was obtained from the National Institute of Health genetic sequence database.

[00176] Oligonucleotides were synthesized by INTEGRATED DNA TECHNOLOGIES (IDT). Probes and primer pairs were chosen for each analyte using OLIGOANALYZER, from IDT. Sense probes containing a fluorophore at the 5' end and a quencher at the 3' end were synthesized for all analytes. All oligonucleotides were lyophilized and reconstituted in TE buffer before use. Tables 9 - 14 show sequence information, target sequence, primers, and probes for each of the six analytes.

Table 9. HIV-1 polyprotease sequence information.

Sequence Information	HIV-1 Poly protease 198mer synthesized from bases 2253 – 2550
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (3' to 5')	GGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGA GTTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTT ATCAAAGTAAGACAGTATGATCAGATACTCATAGAAATCTGTGGACATAA AGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGG
Forward Primer (3' to 5')	GGAAGCTCTATTAGATACAGGAGCAG
Reverse Primer (3' to 5')	CCAATTATGTTGACAGGTGTAGGTCC
Probe 1 (3' to 5')	**/56-FAM/**TGAGTTTGCCAGGAAGATGGAAACCA/3BHQ_1/

Table 10. HIV p17 sequence information.

Sequence Name	HIV-1 P17 199mer synthesized from bases 790 – 1186
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target	CAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATA

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sequence (3' to 5')	TAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAG ACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAG AAAAAAGCACAGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGTCA
Forward Primer (3' to 5')	CAGCTACAACCATCCCTTCAGACA
Reverse Primer (3' to 5')	TGACCTGATTGCTGTGTCCTGTGT
Probe 1 (3' to 5')	**/56-FAM/**AGCAACCCTCTATTGTGTGCATCAAAGG/3BHQ_1
Probe 2 (3' to 5')	**/5Cy3/**AAAGCACAGCAAGCAGCAGCTGA/3BHQ_2/

Table 11. *Plasmodium* ChR7 sequence information.

Sequence Name	*Plasmodium* ChR7 199mer synthesized from bases 1139138 - 1141223
Source	UCSC Plasmodium falciparum Genome Browser Gateway
Target sequence (3' to 5')	GCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAG AGGGAGCCTGAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTA AATTACCCAATTCTAAAGAAGAGAGGTAGTGACAAGAAATAACAATGCAA GGCCAATTTAAAACCTTCCCAGAGTAACAATTGGAGGGCAAGTCTGGTG
Forward Primer (3' to 5')	GCCTAACATGGCTATGACGGGTAA
Reverse Primer (3' to 5')	CACCAGACTTGCCCTCCAATTGTT
Probe 1 (3' to 5')	**/56-FAM/**ATTCCGGAGAGGGAGCCTGAGAAATA/3BHQ_1/
Probe 2 (3' to 5')	**/56-ROXN/**AAGGAAGGCAGCAGGCGCGTAAATTA/3BHQ_2/

Table 12. Herpes simplex virus 2 sequence information.

Sequence Name	HSV-2 193mer synthesized from HSV-2 genome
Source	EMBL Bank AJ303204

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Target sequence (3' to 5')	TCAGCCCATCCTCCTTCGGCAGTATGGAGGGTGTCGCGGCGGCGAGCCGC CGTCCCCAAAGACGTGCGGGTCGTACACGTACACGTACCAGGGCGGCGGG CCTCCGACCCGGTACGCTCTCGTAAATGCTTCCCTGCTGGTGCCGATCTG GGACCGCGCCGCGGAGACATTCGAGTACCAGATCGAACTCGG
Forward Primer (3' to 5')	TCAGCCCATCCTCCTTCGGCAGTAT
Reverse Primer (3' to 5')	CCGAGTTCGATCTGGTACTCGAATGT
Probe 1 (3' to 5')	**/56-FAM/**AAAGACGTGCGGGTCGTACACGTACA/3BHQ_1/
Probe 2 (3' to 5')	**/5Cy5/**TAAATGCTTCCCTGCTGGTGCCGAT/3IAbRQSp/

Table 13. *Mycobacterium tuberculosis* rpoB sequence information

Sequence Name	Mycobacterium Tuberculosis rpoB 200mer synthesized from Mycobacterium tuberculosis rpoB genome
Source	EMBL Bank GQ395623
Target sequence (3' to 5')	GAGTGCAAAGACAAGGACATGACGTACGCGGCCCCGCTGTTCGTCACGGC CGAGTTCATCAACAACAACACCGGCGAGATCAAGAGCCAGACGGTGTTCA TGGGTGACTTCCCGATGATGACCGAGAAGGGCACCTTCATCATCAACGGC ACCGAGCGCGTCGTGGTCAGCCAGCTGGTCCGCTCGCCCGGTGTGTACTT
Forward Primer (3' to 5')	GAGTGCAAAGACAAGGACATGACG
Reverse Primer (3' to 5')	AAGTACACACCGGGCGAGC
Probe 1 (3' to 5')	**/56-FAM/**CGCTGTTCGTCACGGCCGAGTTCAT/3BHQ_1/
Probe 2 (3' to 5')	**/5Cy3/**AGATCAAGAGCCAGACGGTGTTCATG/3BHQ_2/
Probe 3 (3' to 5')	**/5Cy5/**AAGGGCACCTTCATCATCAACGGCA/3IAbRQSp/

Table 14. Dengue virus type 3 sequence information.

Sequence Name	Dengue Virus Type 3 200mer synthesized from Dengue Virus Type 3 genome
Source	GenBank M93130
Target sequence	ATGCCAACTGTGATTGAGCACTTAGAAAGACTACAAAGGAAACATGGAGG

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(3' to 5')	AATGCTTGTGAGAAATCCACTCTCACGAAACTCCACGCACGAAATGTATT GGATATCCAATGGTACAGGCAACATCGTCTCATTCACAATGACACACAGG AGACCCACCATAGAGAAAGATGTGGATTTAGGAGCAGGAACCCGACATGT
Forward Primer (3' to 5')	ATGCCAACTGTGATTGAGCACT
Reverse Primer (3' to 5')	ACATGTCGGGTTCCTGCTCCTAAA
Probe 1 (3' to 5')	**/56-FAM/**ACAAAGGAAACATGGAGGAATGCTTGTGA/3BHQ_1/
Probe 2 (3' to 5')	**/5Cy3/**ACTCTCACGAAACTCCACGCACGAAA/3BHQ_2/
Probe 3 (3' to 5')	**/56-ROXN/**ACAATGACACACAGGAGACCCACCAT/3BHQ_2/
Probe 4 (3' to 5')	**/5Cy5/**GGATATCCAATGGTACAGGCAACATCGT/3IAbRQSp/

3. Polymerase Chain Reactions

[00177] All PCR reactions were performed in a ROCHE 480 LIGHTCYCLER instrument. Forty-five thermal cycles were performed, with a 60 second hot-start at 95°C. The cycling conditions for denaturation, annealing, and extension were 45 seconds, 50 seconds, and 60 seconds at 95°C, 65°C, and 70°C, respectively. Each experiment was run in quintuplicate, with a reaction volume of 15 μL. Fluorescence measurements were obtained in the following channels after every annealing step: 483 nm to 533 nm (FAM), 523 nm to 568 nm (Cy3), 558 nm to 610 nm (ROX), and 615 nm to 670 nm (Cy5). Fluorescence measurements were also obtained after the hot-start and the end of the 45 thermal cycles. The change in the fluorescence intensity between these two measurements (after hot-start and at the end of the 45 cycles) determined the end-point fluorescent signal.

Example 2: Simultaneous Detection of Polynucleotide Markers from Pathogens Based on an Encoding Method Using More Than One Color Per Analyte

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[00178] This example describes the simultaneous detection of up to four polynucleotides selected from HIV (2 polynucleotides), HSV-2, *Mycobacterium*, *Plasmodium*, and dengue virus. Fourteen PCR reactions were assembled, using the reagents described in Table 15.

Table 15. Reagents for 14 PCR reactions.

Reaction No.	Type of Reagent	Reagent	Concentration	Volume
1		UltraPure Water	-	34 µL
		Taq 5x Master Mix	-	42 µL
	Template	Poly Protease Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 nM	2 µL
		Protease RVS Primer	1 nM	2 µL
	Probe	Poly Protease Probe 1	1 nM	2 µL
2		UltraPure Water	-	28 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 nM	2 µL
		Protease RVS Primer	1 nM	2 µL
	Probe	Poly Protease Probe 1	1 nM	2 µL
3		UltraPure Water	-	32 µL
		Taq 5x Master Mix	-	42 µL
	Template	Malaria Template	15 nM	2 µL
	Primers	Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
	Probes	Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
4		UltraPure Water	-	26 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
	Probes	Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
5		UltraPure Water	-	32 µL
		Taq 5x Master Mix	-	42 µL

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	Template	Herpes Template	17 nM	2 µL
	Primers	Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
	Probes	Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
6		UltraPure Water	-	26 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
	Probes	Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
7		UltraPure Water	-	30 µL
		Taq 5x Master Mix	-	42 µL
	Template	TB Template	16 nM	2 µL
	Primers	TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
8		UltraPure Water	-	30 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
9		UltraPure Water	-	28 µL
		Taq 5x Master Mix	-	42 µL
	Template	Dengue Virus Template	17 nM	2 µL
	Primers	Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL

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10		UltraPure Water	-	22 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
11		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease RVS Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
12		UltraPure Water	-	2 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL

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		Malaria RVS Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
13		UltraPure Water	-	12 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
14		UltraPure Water	-	10 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
		Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL

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		Malaria Probe 2	1 μM	2 μL
		Dengue Virus Probe 1	1 μM	2 μL
		Dengue Virus Probe 2	1 μM	2 μL
		Dengue Virus Probe 3	1 μM	2 μL
		Dengue Virus Probe 4	1 μM	2 μL

[00179] Referring to Table 15, reactions 1, 3, 5, 7, and 9 (containing HIV polyprotease, *Plasmodium*, HSV, *Mycobacterium*, and dengue analytes, respectively) were positive controls that provided baseline fluorescence intensity for each polynucleotide analytes alone, in the absence of other analytes. The change in fluorescence intensity in each color was used to provide the expected cumulative intensity level for each color in the chromatogram **(Fig. 3)**, as described more fully below. Reactions 2, 4, 6, 8, and 10 were negative controls used to determine the extent of non-specific amplification. In these reactions, the primers provided were not specific for the analytes provided.

[00180] Experiments 11, 12, 13, and 14 were multiplex detection experiments. Encoding was performed as indicated in Table 16. The measured fluorescence intensity for each assay, in each color, was plotted as black circles in the chromatogram of **Fig. 3.**

Table 16. Coding scheme for detection of pathogens.

	FAM	**Cy3**	**ROX**	**Cy5**
HIV poly-protease	1	0	0	0
HIV p17	1	1	0	0
Plasmodium	1	0	1	0
HSV	1	0	0	1
Mycobacterium	1	1	0	1
Dengue Virus	1	1	1	1

[00181] To construct the chromatogram, the signals of the positive controls (reactions 1, 3, 5, 7, and 9) were used to assemble every possible combination of present sequences, in each color. The cumulative signals were plotted in a diagram, called a "chromatogram" (**Fig. 3**). Cumulative signals corresponding to all possible combinations of the same rank in the same color were organized into their own "band." Doing this for all four colors produced a level and band structure, against which the experimental results of each combination (reactions 11, 12, 13,

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and 14) could be evaluated. **Fig. 3** shows three replicates of a chromatogram constructed using the positive control samples, with experimental results 4112, 3121, and 3102 superimposed on the chromatogram. The experimental results are indicated by black dots.

[00182] The intensity value for each color in reactions 11-14 was measure in arbitrary fluorescence units (AFU), plotted on the chromatogram, and then assigned to a band based on two criteria. First, if an experimental result was within a band, then the result was assigned to the multiplicity of the band (e.g., the Cy3 value of 1 in the left-most chromatogram of **Fig. 3**). Second, if an experimental result was between two bands, a two-step analysis was employed. If the experimental result was between two legitimate results, the experimental result was assigned to the closest band. (e.g., the Cy5 value of 2 in the right-most chromatogram of **Fig. 3**). If the experimental result was between a legitimate result and an illegitimate result, the result was assigned to the band with the legitimate result (e.g., the FAM value of 3 in the middle chromatogram of **Fig. 3**). **Fig. 8** schematically shows a legitimate result of 4112 (top) indicating the presence of HIV polyprotease, *Plasmodium*, herpes simplex, and *Mycobacterium*; and an illegitimate result of 4110 (bottom), which cannot be decoded and indicates a malfunction in the assay.

[00183] Using these criteria, the output of Experiment 12 was designated 4112, which was the correct answer. The output of Experiment 14 could have been designated as 2121 or 3121, but 2121 is illegitimate, so the result must be 3121, which is the correct answer. The output of Experiment 13 could be designated as 3102 or 3101, both of which are legitimate, but the result is clearly closer to 2 than 1 in the Cy5 channel, so 3102, the correct answer, was designated as the result. These results provide experimental validation of the methods of the provided herein.

[00184] The results indicate a trend of slightly lower combined signal than what would be expected from a simple summation of positive-control signals. This may be because the combination of signals is not perfectly linear. This may be a result of additional effects for which the chromatogram construction does not account for at present. These effects may be related to the absolute concentration of quenchers. The higher the absolute concentration of quenchers, the larger the percentage of reaction volume they quench, so that the same percentage of released fluorophores fails to contribute as much as expected to the cumulative signal. One

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solution to this problem is to use low concentrations of probes, so that the percentile loss in signal is negligible, regardless of how much quencher is released.

[00185] **Fig. 3** shows that each rank containing multiple combinations has a relatively wide band. The width of the band is ultimately the difference between the highest and lowest cumulative signals for the positive control samples. If all positive controls in the particular color produced exactly the same fluorescence signal, then the width of each band would be zero. Since instead those signals are somewhat different, the resulting bands are relatively wide. However, there is a simple means to tighten the bands. Instead of loading all probes at the same concentration, as was done for the experiment above, the concentration of each probe can be adjusted so that each of the resulting positive-control signals is the same as the others in the same color. This approach would significantly tighten the bands, making it easier to determine the multiplicity of a signal for a sample containing analytes.

Example 3: Simultaneous Detection of Polynucleotide Markers from Pathogens Based on an Encoding Method Using One Color Per Analyte and Different Intensities

[00186] This example describes the simultaneous detection of up to three polynucleotides selected from dengue virus, HIV p17, and HIV polyprotease. The coding scheme was as depicted in Table 11. As shown in Table 17, each analyte was encoded as a single value of fluorescence intensity in a single color. In this case, as described elsewhere in this disclosure, the coding scheme is non-degenerate by design. This is achieved by assigning an intensity value to each analyte that is equal to the cumulative intensity values for the prior analytes, plus one. Of course, as described elsewhere in the specification, the initial assigned value can be greater than one, for example to better distinguish over noise. Similarly, the values assigned to each analyte can be greater than the cumulative intensity values for the prior analytes plus one, for example, to increase the separation between the intensities and enable more simple assignment of intensities.

Table 17. Coding scheme for detection of dengue virus, HIV polyprotease, and HIV p17.

	FAM
Dengue Virus	1
HIV p17	2

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HIV polyprotease	4

[00187] The same reagents (e.g., templates, primers, and probes) used in Example 2 were used here. The fluorescence signals generated by the respective probes in positive-control end-point PCR reactions were measured and used to calculate the probe concentrations that would produce 1x, 2x, and 4x signal intensities. Experiments were then set up to detect the seven non-null combinations of analyte occurrences. Tables 18-24 provide the PCR reaction components for each of these mixtures.

Table 18. Binary FAM Experiment 1 Cocktail: HIV Polyprotease, HIV p17, and dengue virus

Reagents	Concentration	Volume Added
UltraPure Water	-	50µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	4µL
P17 Template	15nM	4µL
Dengue Virus Template	17nM	4µL
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 19. Binary FAM Experiment 2 Cocktail: dengue virus and HIV p17

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Reagents	Concentration	Volume Added
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	-
P17 Template	15nM	4µL
Dengue Virus Template	17nM	4µL
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 20. Binary FAM Experiment 3 Cocktail: dengue virus and HIV polyprotease

Reagents	Concentration	Volume Added
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	4µL
P17 Template	15nM	-

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Dengue Virus Template	17nM	4µL
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 21. Binary FAM Experiment 4 Cocktail: HIV polyprotease and HIV p17

Reagents	Concentration	Volume Added
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	4µL
P17 Template	15nM	4µL
Dengue Virus Template	17nM	-
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	Concentration	Volume Added

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Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 22. Binary FAM Experiment 5 Cocktail: dengue virus

Reagents	Concentration	Volume Added
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	-
P17 Template	15nM	-
Dengue Virus Template	17nM	4µL
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 23. Binary FAM Experiment 6 Cocktail: HIV p17

Reagents	Concentration	Volume Added
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added

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Poly Protease Template	17nM	-
P17 Template	15nM	4μL
Dengue Virus Template	17nM	-
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1μM	4μL
Poly Protease RVS Primer	1μM	4μL
P17 FWD Primer	1μM	4μL
P17 RVS Primer	1μM	4μL
Dengue Virus FWD Primer	1μM	4μL
Dengue Virus RVS Primer	1μM	4μL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4μL
P17 Probe 1	400nM	4μL
Dengue Virus Probe 1	200nM	4μL

Table 24. Binary FAM Experiment 7 Cocktail: HIV polyprotease

Reagents	Concentration	Volume Added
UltraPure Water	-	58μL
Taq 5x Master Mix	-	25μL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	4μL
P17 Template	15nM	-
Dengue Virus Template	17nM	-
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1μM	4μL
Poly Protease RVS Primer	1μM	4μL
P17 FWD Primer	1μM	4μL
P17 RVS Primer	1μM	4μL
Dengue Virus FWD Primer	1μM	4μL

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Dengue Virus RVS Primer	1μM	4μL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4μL
P17 Probe 1	400nM	4μL
Dengue Virus Probe 1	200nM	4μL

[00188] The total fluorescence signal of each experiment was plotted on a chromatogram (**Fig. 7**) that was constructed as described above. The width of the bands around each positive control measurement is the propagated uncertainty of the 1X measurement. That uncertainty was equated to the standard deviation of the fluorescence signals of the last five PCR cycles in saturation.

[00189] **Fig. 7** shows the results of this experiment. The three right-most results show the measured intensities for dengue virus alone (DEN), HIV p17 alone (P17), and HIV polyprotease (TPP). The assigned intensities for each of these analytes were as indicated in Table 11, and **Fig. 7** confirms that the three analytes yielded the expected intensity results when alone (see DEN, P17, and TPP). At the end of the assay, essentially all of the probes have been hydrolyzed, releasing essentially all of the fluorophores, which emit a signal. A fixed amount of a fluorophore produces a fixed and predictable amount of signal. Therefore a target that is present should generally produce a fixed amount of signal, as determined by the amount of probe added to the reaction volume.

[00190] As expected, the intensity values for the combinations of analytes are equivalent to the sum of the assigned intensities. Since this coding scheme is designed to be non-degenerate, each result can be unambiguously decoded into the presence or absence of a particular analyte. For example, starting from the left side of **Fig. 7**, a result of 7 indicates the presence of all three analytes. A result of 3 indicates the presence of dengue virus and HIV p17 analytes, and the absence of HIV polyprotease. A result of 5 indicates the presence of dengue virus and HIV polyprotease, and the absence of HIV p17. Finally a result of 6 indicates the presence of HIV p17 and HIV polyprotease, and the absence of dengue virus. This coding scheme can be extended to encode additional analytes, as described elsewhere in this disclosure.

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Supplemental Tables and Figures

Table S1 HIV-1 Poly Protease Sequence Information

Sequence Information	HIV-1 Poly protease 198mer synthesized from bases 2253 – 2550
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (5' to 3')	GGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGA GTTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTT ATCAAAGTAAGACAGTATGATCAGATACTCATAGAAATCTGTGGACATAA AGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGG
Forward Primer (5' to 3')	GGAAGCTCTATTAGATACAGGAGCAG
Reverse Primer (5' to 3')	CCAATTATGTTGACAGGTGTAGGTCC
Probe 1 (5' to 3')	/**56-FAM**/TGAGTTTGCCAGGAAGATGGAAACCA/3BHQ_1/

Table S2 HIV-1 P17 Sequence Information

Sequence Name	HIV-1 P17 199mer synthesized from bases 790 – 1186
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (5' to 3')	CAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATA TAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAG ACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAG AAAAAAGCACAGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGTCA
Forward Primer (5' to 3')	CAGCTACAACCATCCCTTCAGACA
Reverse Primer (5' to 3')	TGACCTGATTGCTGTGTCCTGTGT
Probe 1 (5' to 3')	/**56-FAM**/AGCAACCCTCTATTGTGTGCATCAAAGG/3BHQ_1
Probe 2 (5' to 3')	/**5Cy3**/AAAGCACAGCAAGCAGCAGCTGA/3BHQ_2/

Table S3 Malaria ChR7 Sequence Information

Sequence Name	Malaria ChR7 199mer synthesized from bases 1139138 - 1141223
Source	UCSC Plasmodium falciparum Genome Browser Gateway
Target sequence (5' to 3')	GCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAG AGGGAGCCTGAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTA AATTACCCAATTCTAAAGAAGAGAGGTAGTGACAAGAAATAACAATGCAA GGCCAATTTAAAACCTTCCCAGAGTAACAATTGGAGGGCAAGTCTGGTG
Forward Primer (5' to 3')	GCCTAACATGGCTATGACGGGTAA
Reverse Primer (5' to 3')	CACCAGACTTGCCCTCCAATTGTT
Probe 1 (5' to 3')	/**56-FAM**/ATTCCGGAGAGGGAGCCTGAGAAATA/3BHQ_1/

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Probe 2 (5' to 3')	/**56-ROXN**/AAGGAAGGCAGCAGGCGCGTAAATTA/3BHQ_2/

Table S4 Herpes Simplex Virus-2 Sequence Information

Sequence Name	HSV-2 193mer synthesized from HSV-2 genome
Source	EMBL Bank AJ303204
Target sequence (5' to 3')	TCAGCCCATCCTCCTTCGGCAGTATGGAGGGTGTCGCGGCGGCGAGCCGC CGTCCCCAAAGACGTGCGGGTCGTACACGTACACGTACCAGGGCGGCGGG CCTCCGACCCGGTACGCTCTCGTAAATGCTTCCCTGCTGGTGCCGATCTG GGACCGCGCCGCGGAGACATTCGAGTACCAGATCGAACTCGG
Forward Primer (5' to 3')	TCAGCCCATCCTCCTTCGGCAGTAT
Reverse Primer (5' to 3')	CCGAGTTCGATCTGGTACTCGAATGT
Probe 1 (5' to 3')	/**56-FAM**/AAAGACGTGCGGGTCGTACACGTACA/3BHQ_1/
Probe 2 (5' to 3')	/**5Cy5**/TAAATGCTTCCCTGCTGGTGCCGAT/3IAbRQSp/

Table S5 Tuberculosis rpoB Sequence Information

Sequence Name	Mycobacterium Tuberculosis rpoB 200mer synthesized from Mycobacterium tuberculosis rpoB genome
Source	EMBL Bank GQ395623
Target sequence (5' to 3')	GAGTGCAAAGACAAGGACATGACGTACGCGGCCCCGCTGTTCGTCACGGC CGAGTTCATCAACAACAACACCGGCGAGATCAAGAGCCAGACGGTGTTCA TGGGTGACTTCCCGATGATGACCGAGAAGGGCACCTTCATCATCAACGGC ACCGAGCGCGTCGTGGTCAGCCAGCTGGTCCGCTCGCCCGGTGTGTACTT
Forward Primer (5' to 3')	GAGTGCAAAGACAAGGACATGACG
Reverse Primer (5' to 3')	AAGTACACACCGGGCGAGC
Probe 1 (5' to 3')	/**56-FAM**/CGCTGTTCGTCACGGCCGAGTTCAT/3BHQ_1/
Probe 2 (5' to 3')	/**5Cy3**/AGATCAAGAGCCAGACGGTGTTCATG/3BHQ_2/
Probe 3 (5' to 3')	/**5Cy5**/AAGGGCACCTTCATCATCAACGGCA/3IAbRQSp/

Table S6 Dengue Virus Type 3 Sequence Information

Sequence Name	Dengue Virus Type 3 200mer synthesized from Dengue Virus Type 3 genome
Source	GenBank M93130
Target sequence (5' to 3')	ATGCCAACTGTGATTGAGCACTTAGAAAGACTACAAAGGAAACATGGAGG AATGCTTGTGAGAAATCCACTCTCACGAAACTCCACGCACGAAATGTATT

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	GGATATCCAATGGTACAGGCAACATCGTCTCATTCACAATGACACACAGG AGACCCACCATAGAGAAAGATGTGGATTTAGGAGCAGGAACCCGACATGT
Forward Primer (5' to 3')	ATGCCAACTGTGATTGAGCACT
Reverse Primer (5' to 3')	ACATGTCGGGTTCCTGCTCCTAAA
Probe 1 (5' to 3')	/**56-FAM**/ACAAAGGAAACATGGAGGAATGCTTGTGA/3BHQ_1/
Probe 2 (5' to 3')	/**5Cy3**/ACTCTCACGAAACTCCACGCACGAAA/3BHQ_2/
Probe 3 (5' to 3')	/**56-ROXN**/ACAATGACACACAGGAGACCCACCAT/3BHQ_2/
Probe 4 (5' to 3')	/**5Cy5**/GGATATCCAATGGTACAGGCAACATCGT/3IAbRQSp/

Table S7 Experiment 1 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	34µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	2µL
Poly Protease RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1µM	2µL

Table S8 Experiment 2 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	28µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Malaria Template	15nM	2µL
Herpes Template	17nM	2µL
TB Template	16nM	2µL
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	2µL

Poly Protease RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1µM	2µL

Table S9 Experiment 3 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	32µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Malaria Template	15nM	2µL
Primers	**Concentration**	**Volume Added**
Malaria FWD Primer	1µM	2µL
Malaria RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Malaria Probe 1	1µM	2µL
Malaria Probe 2	1µM	2µL

Table S10 Experiment 4 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	26µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Herpes Template	17nM	2µL
TB Template	16nM	2µL
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Malaria FWD Primer	1µM	2µL
Malaria RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Malaria Probe 1	1µM	2µL

Malaria Probe 2	1µM	2µL

Table S11 Experiment 5 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	32µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Herpes Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Herpes FWD Primer	1µM	2µL
Herpes RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Herpes Probe 1	1µM	2µL
Herpes Probe 2	1µM	2µL

Table S12 Experiment 6 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	26µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Malaria Template	15nM	2µL
TB Template	16nM	2µL
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Herpes FWD Primer	1µM	2µL
Herpes RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Herpes Probe 1	1µM	2µL
Herpes Probe 2	1µM	2µL

Table S13 Experiment 7 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	30µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
TB Template	16nM	2µL
Primers	**Concentration**	**Volume Added**
TB FWD Primer	1µM	2µL
TB RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
TB Probe 1	1µM	2µL
TB Probe 2	1µM	2µL
TB Probe 3	1µM	2µL

Table S14 Experiment 8 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	30µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Malaria Template	15nM	2µL
Herpes Template	17nM	2µL
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
TB FWD Primer	1µM	2µL
TB RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
TB Probe 1	1µM	2µL
TB Probe 2	1µM	2µL
TB Probe 3	1µM	2µL

Table S15 Experiment 9 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	28µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Dengue Virus FWD Primer	1µM	2µL
Dengue Virus RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Dengue Virus Probe 1	1µM	2µL
Dengue Virus Probe 2	1µM	2µL
Dengue Virus Probe 3	1µM	2µL
Dengue Virus Probe 4	1µM	2µL

Table S16 Experiment 10 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	22µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Malaria Template	15nM	2µL
Herpes Template	17nM	2µL
TB Template	16nM	2µL
Primers	**Concentration**	**Volume Added**
Dengue Virus FWD Primer	1µM	2µL
Dengue Virus RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Dengue Virus Probe 1	1µM	2µL
Dengue Virus Probe 2	1µM	2µL
Dengue Virus Probe 3	1µM	2µL
Dengue Virus Probe 4	1µM	2µL

Table S17 Experiment 11 Cocktail

Reagents	Concentration	Volume Added
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Malaria Template	15nM	2µL
Herpes Template	17nM	2µL
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	2µL
Poly Protease RVS Primer	1µM	2µL
Malaria FWD Primer	1µM	2µL
Malaria RVS Primer	1µM	2µL
Herpes FWD Primer	1µM	2µL
Herpes RVS Primer	1µM	2µL
Dengue Virus FWD Primer	1µM	2µL
Dengue Virus RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1µM	2µL
Malaria Probe 1	1µM	2µL
Malaria Probe 2	1µM	2µL
Herpes Probe 1	1µM	2µL
Herpes Probe 2	1µM	2µL
Dengue Virus Probe 1	1µM	2µL
Dengue Virus Probe 2	1µM	2µL
Dengue Virus Probe 3	1µM	2µL
Dengue Virus Probe 4	1µM	2µL

Table S18 Experiment 12 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	2µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**

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Poly Protease Template	17nM	2µL
Malaria Template	15nM	2µL
Herpes Template	17nM	2µL
TB Template	16nM	2µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	2µL
Poly Protease RVS Primer	1µM	2µL
Malaria FWD Primer	1µM	2µL
Malaria RVS Primer	1µM	2µL
Herpes FWD Primer	1µM	2µL
Herpes RVS Primer	1µM	2µL
TB FWD Primer	1µM	2µL
TB RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1µM	2µL
Malaria Probe 1	1µM	2µL
Malaria Probe 2	1µM	2µL
Herpes Probe 1	1µM	2µL
Herpes Probe 2	1µM	2µL
TB Probe 1	1µM	2µL
TB Probe 2	1µM	2µL
TB Probe 3	1µM	2µL

Table S19 Experiment 13 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	12µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Herpes Template	17nM	2µL
TB Template	16nM	2µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	2µL

Poly Protease RVS Primer	1μM	2μL
Herpes FWD Primer	1μM	2μL
Herpes RVS Primer	1μM	2μL
TB FWD Primer	1μM	2μL
TB RVS Primer	1μM	2μL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1μM	2μL
Herpes Probe 1	1μM	2μL
Herpes Probe 2	1μM	2μL
TB Probe 1	1μM	2μL
TB Probe 2	1μM	2μL
TB Probe 3	1μM	2μL

Table S20 Experiment 14 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	10μL
Taq 5x Master Mix	-	42μL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2μL
Malaria Template	15nM	2μL
Dengue Virus Template	17nM	2μL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1μM	2μL
Poly Protease RVS Primer	1μM	2μL
Malaria FWD Primer	1μM	2μL
Malaria RVS Primer	1μM	2μL
Dengue Virus FWD Primer	1μM	2μL
Dengue Virus RVS Primer	1μM	2μL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1μM	2μL
Malaria Probe 1	1μM	2μL
Malaria Probe 2	1μM	2μL
Dengue Virus Probe 1	1μM	2μL

Dengue Virus Probe 2	1µM	2µL
Dengue Virus Probe 3	1µM	2µL
Dengue Virus Probe 4	1µM	2µL

Table S21 Binary FAM Experiment 1 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	50µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	4µL
P17 Template	15nM	4µL
Dengue Virus Template	17nM	4µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table S22 Binary FAM Experiment 2 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	-
P17 Template	15nM	4µL
Dengue Virus Template	17nM	4µL

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Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table S23 Binary FAM Experiment 3 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	4µL
P17 Template	15nM	-
Dengue Virus Template	17nM	4µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table S24 Binary FAM Experiment 4 Cocktail

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Reagents	Concentration	Volume Added
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	4µL
P17 Template	15nM	4µL
Dengue Virus Template	17nM	-
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table S25 Binary FAM Experiment 5 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	-
P17 Template	15nM	-
Dengue Virus Template	17nM	4µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL

Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table S26 Binary FAM Experiment 6 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	-
P17 Template	15nM	4µL
Dengue Virus Template	17nM	-
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table S27 Binary FAM Experiment 7 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**

Poly Protease Template	17nM	4µL
P17 Template	15nM	-
Dengue Virus Template	17nM	-
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Fig. 5A

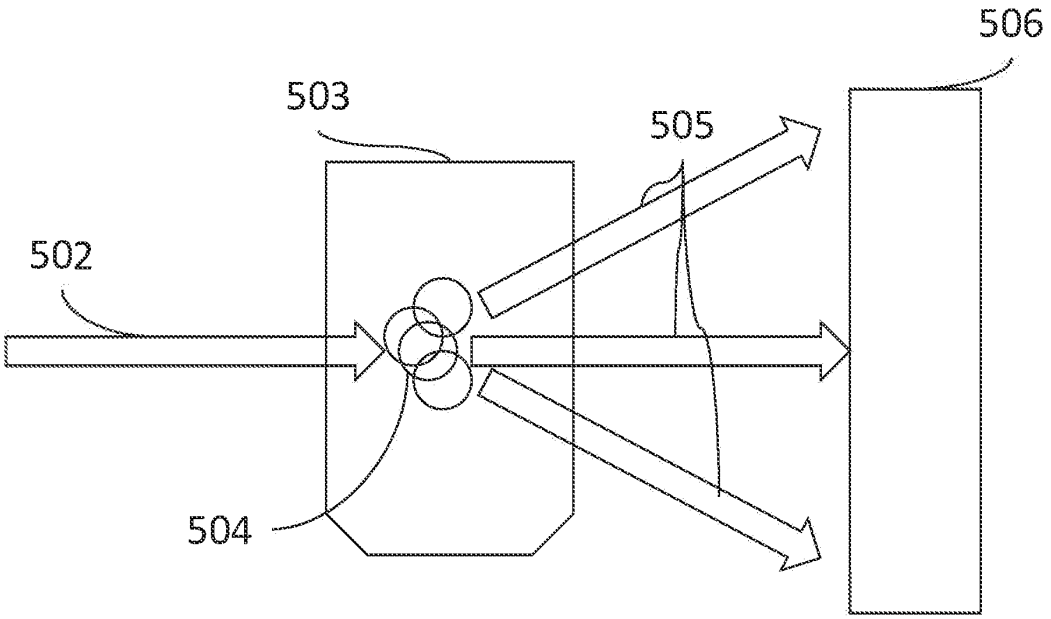
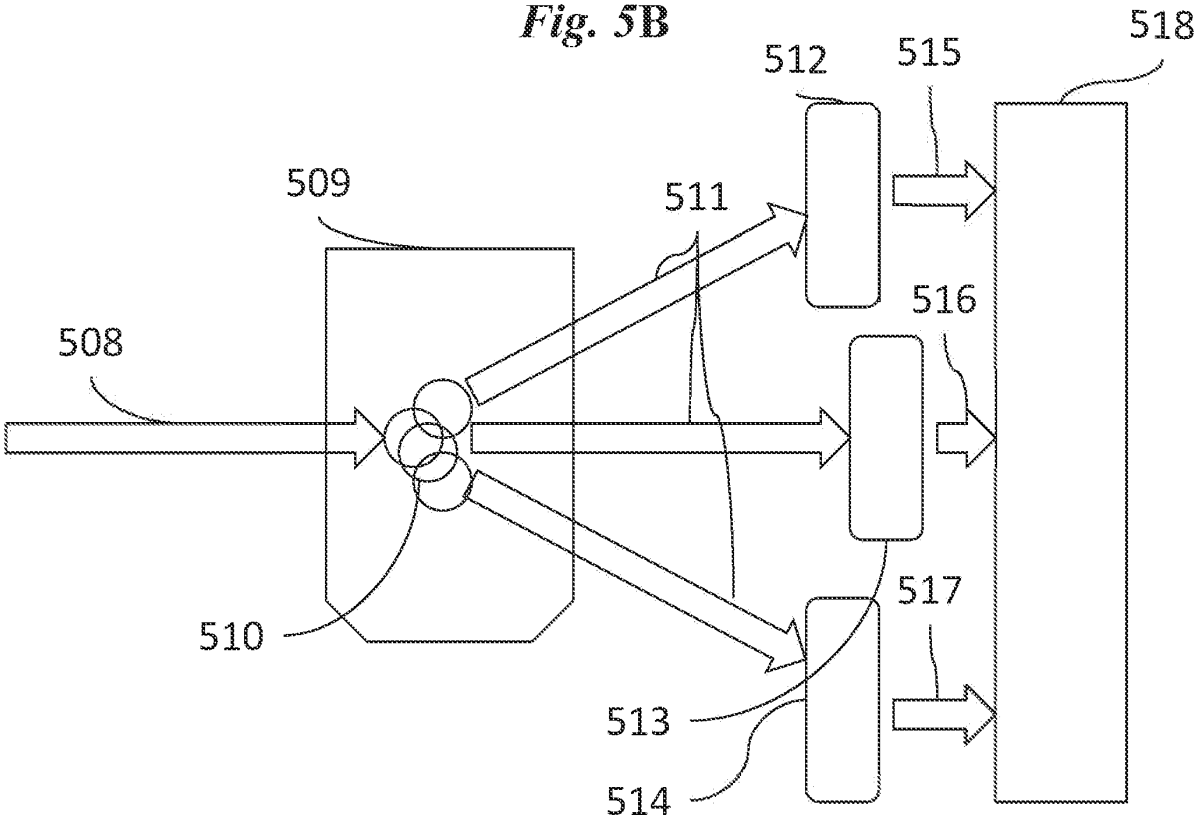


Fig. 5B



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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/352,112	**11/12/2024 06:09:39 PM Z ET**	**88DG-373535-US**

Title of Invention

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	3145	FILED BY	Annie Wong
PATENT CENTER #	67938145	FILING DATE	07/13/2023
CUSTOMER #	30764	FIRST NAMED INVENTOR	Emil P. KARTALOV
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	David Heisey

Documents

TOTAL DOCUMENTS: 4

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
312_Amendment_18352112.pdf	3	Amendment after Notice of Allowance (Rule 312)	36 KB
Replacement_Drawing_18352112-.pdf	1	Drawings-only black and white line drawings	324 KB
Substitute_Spec_marked-up_18352112-.pdf	91	Specification	466 KB
Substitute_Spec_clean_18352112-.pdf	91	Specification	464 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)

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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 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.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION

Title:

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

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Attorney Docket No.: 88DG-373535-US (CIT-6088-US6)

Sheets of Drawings: 8

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of U.S. Application No. 16/937,464, filed on July 23, 2020, which is a continuation of U.S. Application No. 15/914,356, filed on March 7, 2018, issued as U.S. Patent No. 10,770,170, which is a continuation of U.S. Application No. 14/451,876, filed on August 5, 2014, issued as U.S. Patent No. 10,068,051, which is a continuation of U.S. Application No. 13/756,760, filed on February 1, 2013, issued as U.S. Patent No. 8,838,394, which claims the benefit of U.S. provisional applications 61/594,480, filed February 3, 2012 and 61/703,093, filed September 19, 2012, each of which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

[0002] This application contains a Sequence Listing which has been submitted electronically in XML format and is incorporated by reference in its entirety. The XML copy, created on July 12, 2023, is named 373535.xml and is 57,570 bytes in size.

GOVERNMENT RIGHTS

[0003] This invention was made with government support under Grant No. 1144469 awarded by the National Science Foundation. The government has certain rights in the invention.

BACKGROUND

[0004] Multiplexed reactions offer significant advantages over traditional uniplex reactions, including performance of parallel reactions on the same sample, use of the same chamber to perform multiple reactions, and the ability to extract rich information from a sample in a fast and efficient manner. However, to achieve these benefits, multiplexed assays generally require complex reporting mechanisms, namely spectrally resolved fluorescence or chemiluminescence (e.g., PCR, ELISA), spatially resolved signals (e.g., microarrays, gel electrophoresis), temporally resolved signals (e.g., capillary electrophoresis), or combinations thereof (e.g., Sanger

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sequencing). There is a need for multiplexed reactions that can be carried out in a single solution.

SUMMARY OF THE INVENTION

[0005] This disclosure provides methods, compositions, systems, and kits for the multiplexed detection of analytes. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some examples, each of the analytes is encoded as a value of one (or at least one) component of a signal.

[0006] In some examples, an assay provided in this disclosure is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = \log_2 (F + 1)$ and F is the maximum cumulative value of one component of a signal (e.g., an intensity) when all the analytes are present.

[0007] In some cases, each analyte is encoded as at least one first value in a first component of a signal (e.g., at least one intensity or range of intensities) and at least one second value in a second component of a signal (e.g., at least one wavelength or range of wavelengths). In some examples, each analyte is encoded as a first value in a first component of a signal at each of a plurality of second values in a second component of a signal (e.g., a signal intensity or range of signal intensities at each of a plurality of wavelengths or ranges of wavelengths).

[0008] In some examples, an assay provided herein is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = C * \log_2 (F + 1)$, where C is the number of the second values used to encode the analytes and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present.

[0009] In some cases, an assay provided in this disclosure is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = (P * T) + 1$, where P is the number of codes per tier in a coding scheme and T is the number of tiers. In some cases, the number of tiers $T = \log_4 (F + 1)$, and F is the maximum cumulative value of a first component of a signal, for any second value, when all of the analytes are present.

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[0010] In some examples, the first value is an intensity or range of intensities. In some cases, a coding scheme comprises at least three intensities or ranges of intensities.

[0011] In some cases, the second value is a wavelength or range of wavelengths. In some examples, the coding scheme comprises at least five wavelengths or ranges of wavelengths.

[0012] In some examples, the signal is an electromagnetic signal. In some cases, the electromagnetic signal is a fluorescence emission signal. In some examples, the intensity of the fluorescence emission signal is measured at at least four wavelengths or ranges of wavelengths.

[0013] In some cases, an assay provided herein is performed with reagents that are lyophilized prior to use.

[0014] In some cases, a detecting step is performed with reagents comprising hybridization probes. In some examples, the number of the hybridization probes is greater than the number of analytes. In some cases, the set of hybridization probes comprise one or more hybridization probes specific for different analytes and comprising an identical fluorophore or combination of fluorophores. In some examples, a sample is contacted with at least 18 of said hybridization probes. In some examples, a detecting step is performed with reagents comprising at least one pair of primers. In some cases, at least one pair of primers are capable of amplifying a region complementary to at least three of said hybridization probes.

[0015] In some examples, the signal that is measured is generated during a polymerase chain reaction. In some cases, the polymerase chain reaction is selected from the group consisting of an end-point polymerase chain reaction, a real-time polymerase chain reaction, a digital polymerase chain reaction, and combinations thereof.

[0016] In some examples, at least one cumulative measurement is performed on a solution.

[0017] In some examples, at least one analyte is encoded by at least one additional value (i.e., at least two values together), wherein the at least one additional value is selected from the group consisting of a value from at least one additional component of a signal, a value from at least one component of a different signal, and combinations thereof. For example, the at least one additional value may be selected from the group consisting of a fluorescence emission intensity, a fluorescence emission wavelength, a Förster resonance energy transfer (FRET) emission intensity, a FRET emission wavelength, an electrochemical signal, a chemiluminescence

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wavelength, a chemiluminescence intensity, a fluorescence bleaching rate, a chemiluminescence bleaching rate, and combinations thereof.

[0018] In some cases, a chromatogram is constructed. The chromatogram may be constructed by plotting all possible combinations of first values and second values for positive control samples for each analyte.

[0019] In some examples, at least one of the analytes comprises a polynucleotide. In some cases, the polynucleotide is from a source selected from the group consisting of an animal, a plant, a bacteria, a fungus, a parasite, and a virus. In some examples, the polynucleotide is from a source selected from the group consisting of human immunodeficiency virus, herpes simplex virus, human papilloma virus, Plasmodium, Mycobacterium, dengue virus, hepatitis virus, and influenza virus. In some cases, the polynucleotide is selected from the group consisting of human immunodeficiency virus polyprotease, human immunodeficiency virus p17, human papilloma virus E6, and human papilloma virus E7.

[0020] In some cases, a sample is selected from the group consisting of a clinical sample, a food sample, an environmental sample, a pharmaceutical sample, and a sample from a consumer product.

[0021] In some examples, information concerning the presence or absence of an analyte is transmitted through a computer network.

[0022] In some cases, information concerning the presence or absence of an analyte is provided to a physician. In some examples, a clinical decision is made based on such information.

[0023] In some examples, at least one step of a method provided herein is performed using instructions on a computer readable medium. In some cases, the instructions are located on a remote server. In some examples, the instructions are located on a thermal cycler. In some cases, the instructions are located on a computer in communication with a thermal cycler.

[0024] In some cases, at least one of the analytes is a positive control analyte.

[0025] In some examples, the coding scheme is non-degenerate. In some cases, the coding scheme is designed to be non-degenerate. In some examples, the coding scheme is made non-degenerate by enumerating every legitimate result that can be obtained from the coding scheme, identifying each legitimate result that is degenerate, and eliminating at least one potential analyte code from the coding scheme to eliminate degeneracy.

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[0026] In some cases, an assay provided herein is an end-point assay. In some examples an assay provided in this disclosure is ended at a threshold number of cycles set by the limit of detection of an instrument.

[0027] In certain examples, an assay provided herein is a liquid phase assay.

[0028] In some cases, an assay provided herein is quantitative.

[0029] In some cases, this disclosure provides a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

[0030] In some examples, the method described in the preceding paragraph is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = \log_2 (F + 1)$ and F is the maximum cumulative value of the first values when all of the analytes are present. In some cases, the first value is an intensity or range of intensities. In some examples, the first value has a minimum value in the coding scheme that is selected from the group consisting of at least 1, at least 2, at least 4, at least 8, at least 16, at least 32, and at least 64. In some cases, the first value is incremented in the coding scheme by an amount equal to the cumulative maximum of said preceding first values plus one. In other cases, the first value is incremented in the coding scheme by an amount greater than the cumulative maximum of the preceding first values plus one.

[0031] In some cases, this disclosure provides a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding

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scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said at least one first value and at least one said second value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme, wherein, when degeneracy is eliminated, said method is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

[0032] In some cases, each of the analytes of the method described in the preceding paragraph is encoded as a first value in a first component of the signal at each of a plurality of second values in a second component of the signal. In some cases, when degeneracy is eliminated, this method is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single volume using four second values. In some examples, when degeneracy is eliminated, the coding scheme comprises T non-degenerate tiers, wherein $T = \log_4 (F + 1)$ and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present. In some cases, when degeneracy is eliminated, the method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = (P * T) + 1$ and P is the number of codes per tier. In some examples, when degeneracy is eliminated, the method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = C * \log_2 (F + 1)$, C is the number of second values in the coding scheme, and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present.

[0033] This disclosure also provides non-degenerate coding schemes capable of unambiguously encoding the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis.

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[0034] In some examples, a non-degenerate coding scheme of this disclosure is generated by a method comprising: (a) generating a code for each potential analyte, wherein each potential analyte is encoded by at least one value of at least one component of a signal; (b) enumerating every legitimate cumulative result for all possible combinations of presence or absence of each analyte; (c) identifying each legitimate result that is degenerate; and (d) eliminating at least one code to eliminate degeneracy. In some cases, the coding scheme may be expanded by adding additional codes that are at least one unit greater than the sum of all previous codes in the at least one component of said signal. In some cases a coding scheme is generated by a method of mathematical iteration that guarantees non-degeneracy by construction.

[0035] In some cases, this disclosure provides systems for detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

[0036] In some cases, this disclosure provides systems for detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values and said second values within said sample, thereby

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providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme, wherein, when degeneracy is eliminated, said system is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

[0037] In some cases, this disclosure provides methods of detecting the presence or absence of each analyte of a plurality of analytes for a third party, comprising: (a) obtaining the identity of each of said analytes from a party; (b) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (c) providing said party with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present, said party: (i) contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and (ii) cumulatively measuring said first values within said sample, thereby providing a cumulative measurement; and (d) obtaining said cumulative measurement from said party; (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and (f) providing said party with information about the presence or absence of each of said analytes.

[0038] In some cases, this disclosure provides methods of detecting the presence or absence of each analyte of a plurality of analytes for a third party, comprising: (a) obtaining the identity of each of said analytes from a party; (b) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (c) providing said party with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present, said party: (i) contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and (ii) cumulatively measuring said first values and said second values within said

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sample, thereby providing a cumulative measurement; and (d) obtaining said cumulative measurement from said party; (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and (f) providing said party with information about the presence or absence of each of said analytes.

[0039] In some cases, this disclosure provides compositions for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising a first value, wherein said reagents are capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

[0040] In some cases, this disclosure provides compositions for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said reagents are capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

[0041] In some cases, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising a first value, wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

[0042] In some examples, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

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[0043] In some cases, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising a kit body (601), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (602), a bottle comprising primers for amplification (603), and a bottle comprising reagents for amplification (604), wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

[0044] In some examples, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, a kit body (601), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (602), a bottle comprising primers for amplification (603), and a bottle comprising reagents for amplification (604), each probe generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said probes generates only one second value.

INCORPORATION BY REFERENCE

[0045] 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.

BRIEF DESCRIPTION OF THE DRAWINGS

[0046] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0047] Fig. 1 shows a comparison between a traditional encoding method of detecting four analytes with four colors and an encoding method of the invention able to detect 16 or more sequences with four colors. Colors are indicated by B, G, Y, and R, which indicate blue, green, yellow, and red, respectively.

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[0048] Fig. 2 shows a schematic representation of detection of six analytes (including control) with four colors. The analytes are detected using hybridization probes attached to a fluorophore (B, G, Y, R; blue, green, yellow, and red, respectively) and a quencher (oval with "X"). The control sequence and the five other sequences (Dengue Fever = dengue virus; Tuberculosis = *Mycobacterium tuberculosis*; P17 = HIV p17; Malaria = *Plasmodium falciparum;* and Herpes = herpes simplex virus 2) are all detected using a probe labeled with a blue fluorophore. The non-control analytes are each detected using 1-3 additional probes. For example, the dengue virus analyte is detected using three additional probes with green, yellow, and red fluorophores; the herpes simplex virus 2 analyte is detected using one additional probe with a red fluorophore; and so on.

[0049] Fig. 3 shows chromatograms of experimental results obtained as described in Example 2.

[0050] Fig. 4 shows an exemplary embodiment of the invention in which a computer is used to perform one or more steps of the methods provided herein.

[0051] Figs. 5A and **5B** show schematics of two different methods of detecting wavelength and intensity of a light signal, such as a fluorescence emission signal.

[0052] Fig. 6 shows a schematic of exemplary kits.

[0053] Fig. 7 shows chromatograms of experimental results obtained as described in Example 3.

[0054] Fig. 8 shows a schematic of a legitimate result (top) and an illegitimate result (bottom).

DETAILED DESCRIPTION OF THE INVENTION

[0055] Fluorescence detection has been a preferred technique for multiplexed assays because of several desirable features, including compatibility with biochemical assays, the relatively small size of fluorescent labels, simple means of conjugation to molecules of interest, affordability, low toxicity, stability, robustness, detectability with inexpensive optics, and an ability to be combined with spatial arrays. However, standard fluorophores have wide emission spectra. Therefore, in order to avoid spectral overlap (i.e., to preserve spectral resolution) only a relatively small number of colors (e.g., 4 to 6) are typically used simultaneously in multiplexed fluorescent assays.

[0056] The traditional encoding method for multiplexed fluorescent assays has been to encode each analyte with a single color; i.e., M=N, where M is the number of analytes that can be

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detected and N is the number of spectrally resolved fluorescent probes. Whenever higher factors of multiplexing are required (i.e., M>N), fluorescence is generally combined with other techniques, such as aliquoting, spatial arraying, or sequential processing. These additional processing steps are labor-intensive and frequently require relatively expensive and complex optical and mechanical systems (e.g., spectrometers, mechanized microscopy stages, microfluidics, droplet generators, scanners, and the like). Such systems are often impractical to deploy in certain settings, particularly point-of care and low-resource settings. Thus, there is a significant need for multiplexed encoding and decoding methods that can provide an inexpensive means of multiplexing while avoiding the use of expensive additional processing steps.

[0057] This disclosure provides methods, systems, compositions, and kits for the detection of multiple analytes in a sample. Analytes are detected based on the encoding, analysis, and decoding methods presented herein. In some examples, each analyte to be detected is encoded as a value of a signal (e.g., intensity), where the values are assigned so that the results of the assay unambiguously indicate the presence or absence of the analytes being assayed. In other examples, each analyte to be detected is encoded as a value in each of at least two components of a signal (e.g., intensity and wavelength). The at least two components of a signal may be orthogonal. Similarly, as described more fully elsewhere in this disclosure, multiple orthogonal signals may be used, such as a combination of a fluorescent signal and an electrochemical signal. The analyte may be any suitable analyte, such as a polynucleotide, a protein, a small molecule, a lipid, a carbohydrate, or mixtures thereof. The signal may be any suitable signal such as an electromagnetic signal, a light signal, a fluorescence emission signal, an electrochemical signal, a chemiluminescent signal, and combinations thereof. The at least two components of the signal may be any suitable two components, such as an amplitude and a frequency or an intensity and a wavelength.

[0058] After encoding of the analytes, a sample is provided wherein the sample comprises or may comprise at least one of the encoded analytes. The sample is contacted with an analyte-specific reagent or reagents that generate a particular signal, as specified for each analyte in the coding scheme, in the presence of an analyte. A reagent may be any suitable reagent that is capable of generating such a signal in the presence of its corresponding analyte, for example, an oligonucleotide probe attached to a fluorophore and a quencher (e.g., a TAQMAN probe). If the

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reagent is an oligonucleotide probe attached to a fluorophore and a quencher, a nucleic acid amplification may be performed to generate the signal.

[0059] After addition of the reagent(s), the signal is quantified. In some cases, this quantification is performed by measuring one component of the signal (e.g., fluorescence intensity) and determining the presence and absence of certain analytes based on the values used to encode the presence of each analyte and the cumulative value of the signal.

[0060] In some cases, at least two components of a signal (e.g., intensity and wavelength) are cumulatively measured for the sample. This measurement can be performed, for example, by measuring the intensity at a particular wavelength or the intensity within a particular range of wavelengths. The presence or absence of an analyte may then be determined based on the values of each of the at least two components of the signal and the values used to encode the presence of the analyte (i.e., those values in the coding scheme).

[0061] The encoding may be performed in a manner that reduces or eliminates the number of possible degenerate (e.g., ambiguous) results that can be obtained by the method. As described elsewhere in this specification, the full coding capability of a particular coding scheme may be enumerated, and certain potential analyte codes may be eliminated from the coding scheme to reduce or eliminate degeneracy. Similarly, a coding scheme may be designed to be non-degenerate, so that a reduction or elimination of degeneracy is not necessary. A decoding matrix may be constructed to translate cumulative signal measurements (e.g., intensities or intensities at particular wavelengths) into the presence or absence of certain analytes, corresponding to the constituent signals of the cumulative signal measurement.

I. Definitions

[0062] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

[0063] As used herein, the singular forms “a,” “an,” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms “including,” “includes,” “having,” “has,” “with,” “such as,” or variants thereof, are used in either the specification and/or the claims, such terms are not limiting and are intended to be inclusive in a manner similar to the term “comprising”.

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[0064] The term "about," as used herein, generally refers to a range that is 15% greater than or less than a stated numerical value within the context of the particular usage. For example, "about 10" would include a range from 8.5 to 11.5.

[0065] The terms "dimension" and "component," as used herein when referring to a signal, generally refer to an aspect of the signal that may be quantified. For example, if a signal is generated by a fluorescent molecule, it may be quantified in terms of its wavelength (e.g., a first dimension or component) and its intensity (e.g., a second dimension or component).

[0066] The term "decoding," as used herein, generally refers to a method of determining which analytes are present based on the cumulative signal and a coding scheme or decoding matrix that enables the conversion of a cumulative signal to information concerning the presence or absence of one or more analytes.

[0067] The term "encoding," as used herein, generally refers to the process of representing an analyte using a code comprising values of a signal, such as intensity, or values in each of at least two components of a signal or signals, such as wavelength and intensity.

[0068] The terms "oligonucleotide probe attached to a fluorophore and a quencher" and "TAQMAN probe" generally refer to hydrolysis probes used to detect the presence of an analyte in a polynucleotide amplification assay. These probes comprise an oligonucleotide probe attached to a fluorophore and a quencher. So long as the quencher and the fluorophore are in proximity, the quencher quenches the fluorescence emitted by the fluorophore upon excitation by a light source. The sequence of the oligonucleotide probe is designed to be complementary to a polynucleotide sequence present in an analyte, and therefore capable of hybridizing to the polynucleotide sequence present in the analyte. Hybridization of the oligonucleotide probe is performed in a nucleic acid amplification reaction comprising primers (e.g., a polymerase chain reaction). Upon extension of the primers by a DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and the quencher into the medium. The proximity between the fluorophore and the quencher is broken and the signal from the fluorophore is no longer quenched. Thus, the amount of fluorescence detected is a function of the amount of analyte present. If no analyte is present, the probe will not hybridize to an analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be produced.

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[0069] The term "orthogonal," as used herein, generally refers to at least two components of a signal (e.g., wavelength and intensity), or at least two different signals (e.g., fluorescence emission and electrochemical signal), that can be varied independently or approximately independently. For example, wavelength and intensity are considered orthogonal or approximately orthogonal when fluorescent molecules are used. Among other factors, the wavelength of fluorescence emission will depend on the composition of the fluorescent molecule and the intensity of the fluorescence will depend on the amount of molecule present. Although wavelength and intensity are examples of two components of a signal that can be varied approximately independently, the methods described herein are not limited to components that can be varied independently or approximately independently. Components of a signal, or signals, that vary non-independently may also be used, so long as the components or signals are characterized well enough to enable encoding, measurement, and decoding. For example, if the variance in one component or signal affects the variance in another component or signal, the two components or signals may still be used so long as the relationship between the variances is understood.

[0070] The terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides. Exemplary polynucleotides include deoxyribonucleic acids, ribonucleic acids, and synthetic analogues thereof, including peptide nucleic acids.

[0071] The term "probe," as used herein, generally refers to a reagent capable of generating a signal in the presence of a particular analyte. A probe generally has at least two portions: a portion capable of specifically recognizing an analyte, or a portion thereof, and a portion capable of generating a signal in the presence of an analyte, or a portion thereof. A probe may be an oligonucleotide probe attached to a fluorophore and a quencher, as described above and elsewhere in this disclosure. A probe may also be any reagent that generates a signal in the presence of an analyte, such as an antibody that detects an analyte, with a fluorescent label that emits or is quenched upon binding of the antibody to an analyte. Any suitable probe may be used with the methods presented in this disclosure, so long as the probe generates a quantifiable signal in the presence of an analyte. For example, the analyte-specific portion of a probe may be coupled to an enzyme that, in the presence of an analyte, converts an uncharged substrate into a

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charged product, thereby increasing the electrical conductivity in the medium over time. In this case, different analytes may be encoded by coupling the analyte-specific portion of the probe (e.g., hybridization probe or antibody) to an enzyme at different ratios. The resulting rate of increased conductivity in the medium will be cumulative for all analytes present in the medium. Encoding analytes according to the methods provided herein enables conversion of the conductivity measurements into unambiguous (i.e., non-degenerate) results providing information about the presence or absence of particular analytes. Similarly, a probe may comprise an enzyme producing a chemiluminescent product from a substrate. The amount of chemiluminescence may then be used to encode the presence of particular analytes.

II. Encoding and Decoding Methods

A. Traditional Fluorescent Encoding and Decoding Method

[0072] A commonly used method of determining the presence of an analyte uses four spectrally resolved fluorescent molecules to indicate the presence or absence of four analytes. An example of this method is presented on the left-hand side of **Fig. 1**. The left-hand side of **Fig. 1** shows an encoding method where four analytes (Seq 1, Seq 2, Seq 3, and Seq 4) are each encoded by a single color (blue, green, yellow, and red, respectively). The color represents a fluorophore attached to an oligonucleotide probe that also comprises a quencher. In the system shown on the left-hand side of **Fig. 1**, there are four different oligonucleotide probes, each comprising a single fluorophore (blue, green, yellow, or red) and a quencher. The presence or absence of an analyte is determined based on the presence or absence of a signal in a particular color.

[0073] The chart on the left-hand side of **Fig. 1** shows intensity versus color for a hypothetical sample containing two analytes: Seq 1 and Seq 3. The presence of these analytes is determined based on the measurement of a blue signal (corresponding to Seq 1) and a yellow signal (corresponding to Seq 3). The absence of Seq 2 and Seq 4 is indicated by the absence of a blue and red signal.

[0074] Table 1 shows a translation of this coding scheme into a binary format. Each analyte is encoded as a value in each of two components of a fluorescent signal: (1) color (also known as wavelength; or range of wavelengths) and (2) intensity (indicated by the numbers within the table). For example, A (e.g., Seq 1) has a color of blue and an intensity of 1; B (e.g., Seq 2) has

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a color of green and an intensity of 1; and so on. The intensity of the signal within each color range may be quantified as described herein, for example by measuring the intensity of the signal within a particular wavelength range determined by a band pass filter. A result of 1000 indicates that only analyte A is present; a result of 1100 indicates that analytes A and B are present; and so on.

Table 1. Traditional encoding of four analytes with four probes, each probe having a single color, and one probe per analyte.

	Blue	**Green**	**Yellow**	**Red**
A	1	0	0	0
B	0	1	0	0
C	0	0	1	0
D	0	0	0	1

B. Encoding Methods Using More Than One Color Per Analyte

[0075] The traditional method described above suffers from the fact that it is limited by the number of spectrally resolvable fluorophores. More specifically, the number of detectable analytes is equal to the number of spectrally resolvable fluorophores. Therefore, the number of analytes may only be increased by increasing the number of spectrally resolvable fluorophores.

[0076] This disclosure provides methods that overcome this limitation. More specifically, in some cases, by utilizing at least two components of a signal during encoding, the methods described herein may be used to detect more than one analyte per fluorophore. For example, using the method provided herein 1.5, 1.6, 1.7, 1.8, 1.9, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes may be detected per fluorophore. In some cases, the methods provided herein may be used to detect at least 1.5, 1.6, 1.7, 1.8, 1.9, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes may be detected per fluorophore. In some cases, the methods provided herein may be used to detect 1.5-2, 2-4, 1.5-4, 4-6, 2-6, 6-10 analytes per fluorophore.

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[0077] In some cases, the methods provided in this disclosure may include the use of a control color. The control color may be attached to one or more probes binding a positive control analyte, and each analyte to be detected, in a sample. If the same sequence occurs in the positive control analyte and each analyte to be detected, a single control probe may be used. If the same sequence does not occur in the positive control analyte and each analyte to be detected, different probes may be used, but each probe may still be attached to the control color.

[0078] For example, building on the traditional methods described above, one color (e.g., blue) may be used to encode the presence of a control analyte that is always present in the sample. The control analyte may be added to the sample, or may be inherently present in the sample. The remaining colors (e.g., green, yellow, red) may be used to encode the presence of additional analytes. Table 2 shows an example of one such method.

Table 2. Encoding of four analytes, including control, with four colors and up to two colors per analyte.

	Blue	**Green**	**Yellow**	**Red**
Ctrl. (p)	1	0	0	0
A	1	0	0	1
B	1	0	1	0
C	1	1	0	0

[0079] In Table 2, the presence of the control (p) is indicated by a result of 1000. The presence of the control and analyte A is indicated by a result of 2001. The presence of the control and all three other analytes is indicated by a result of 4111. The control color (blue) provides an indication that the assay is functioning properly. The intensity of the blue color reports the number of analytes that are present in a test sample. Of course, any color may be used as the control color. One of skill in the art will recognize that certain practical considerations might make it preferable to use one color over another as the control color. For example, if one color is better detected in a particular optical system (or system of fluorophores), it might be practically preferable to use that color as a control color.

[0080] The coding scheme shown in Table 2 encodes each analyte using one control color and one additional color (up to 2 probes per analyte). However, as shown below, the number of

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analytes that can be encoded increases when up to 4 colors are used per analyte. Table 3 shows an exemplary coding scheme where each analyte is encoded by up to 4 colors. In the scheme shown in Table 3, the control color (blue) is generated in the presence of the control sequence and each of the other seven analytes (A-G). The other seven analytes are each encoded by the presence of one to three additional colors. The colors may be contained on different probes (e.g., oligonucleotide probes attached to a fluorophore and a quencher) or on the same probe (which can have multiple fluorophores and multiple quenchers).

Table 3. Encoding of eight analytes (including control) with four colors and up to four colors per analyte.

	Blue	**Green**	**Yellow**	**Red**
Ctrl. (p)	1	0	0	0
A	1	0	0	1
B	1	0	1	0
C	1	1	0	0
D	1	0	1	1
E	1	1	0	1
F	1	1	1	0
G	1	1	1	1

[0081] In Table 3, the presence of the control and all seven other analytes is indicated by a result of 8444. Three analytes (A, B, and C) are encoded by two colors. Three analytes (D, E, and F) are encoded by three colors, and one analyte (G) is encoded by four colors. The presence of the control (p) is indicated by a result of 1000. The presence of the control and analyte A is indicated by a result of 2001. The presence of the control and analyte G is indicated by a result of 2111. These results express the cumulative intensity of the signal in each color. For example, the result 2111 has a 2X signal intensity in the blue channel, while the result 1000 has only a 1X signal intensity in the blue channel.

[0082] Using Table 3, each of the possible cumulative assay results can be enumerated, in terms of color (blue, green, yellow, red) and intensity (0-8). Table 4 shows a "decoding matrix" generated by enumerating each of the possible cumulative assay results based on the encoding method presented in Table 3 and providing the corresponding decoded result of each assay in terms of the analyte(s) present in the sample. A similar decoding matrix may be generated for

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any coding scheme described herein, by enumerating each of the possible cumulative assay results based on the coding scheme and providing the corresponding decoded result of each assay in terms of the analyte(s) present in (or absent from) the sample. In some cases, as described below, one or more analytes may be removed from the coding scheme in order to reduce or eliminate degeneracy.

Table 4. Decoding matrix for encoding method presented in Table 3.

Cumulative Assay Result				
Blue	**Green**	**Yellow**	**Red**	**Analyte(s) Present**
1	0	0	0	p
2	0	0	1	pA
2	0	1	0	pB
2	1	0	0	pC
2	0	1	1	pD
2	1	0	1	pE
2	1	1	0	pF
2	1	1	1	pG
3	0	1	1	pAB
3	1	0	1	pAC
3	0	1	2	pAD
3	1	0	2	pAE
3	1	1	1	pAF, pCD, pBE
3	1	1	2	pAG, pDE
3	1	1	0	pBC
3	0	2	1	pBD
3	1	2	0	pBF
3	1	2	1	pBG, pDF
3	2	0	1	pCE
3	2	1	0	pCF
3	2	1	1	pCG, pEF
3	1	2	2	pDG
3	2	1	2	pEG
3	2	2	1	pFG
4	1	1	1	pACB
4	0	2	2	pABD
4	2	2	0	pBCF
4	2	0	2	pACE
4	1	1	2	pABE, pACD
4	1	2	1	pABF, pBCD
4	2	1	1	pACF, pBCE

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4	1	2	2	pADF, pABG, pBDE
4	2	1	2	pACG, pCDE, pAEF
4	2	2	1	pBCG, pBEF, pCDF
4	2	2	2	pCDG, pAFG, pBEG, pDEF
4	1	1	3	pADE
4	1	3	1	pBDF
4	3	1	1	pCEF
4	1	2	3	pADG
4	3	2	1	pCFG
4	2	1	3	pAEG
4	2	3	1	pBFG
4	1	3	2	pBDG
4	3	1	2	pCEG
4	2	2	3	pDEG
4	3	2	2	pEFG
5	1	2	2	pABCD
5	2	1	2	pABCE
5	2	2	1	pABCF
5	2	2	2	pABCG, pABEF, pBCDE, pACDF
5	1	2	3	pABDE
5	2	1	3	pACDE
5	1	3	2	pABDF
5	2	1	3	pBCDF
5	3	1	2	pACEF
5	3	2	1	pBCEF
5	2	2	3	pABEG, pACDG, pADEF
5	2	3	2	pABFG, pBCDG, pBDEF
5	3	2	2	pACFG, pBCEG, pCDEF
5	1	3	3	pABDG
5	3	1	3	pACEG
5	3	3	1	pBCFG
5	2	3	3	pADFG, pBDEG
5	3	3	2	pBEFG, pDEFG
5	3	2	3	pCDEG, pAEFG
5	2	2	4	pADEG
5	2	4	2	pBDFG
5	4	2	2	pCEFG
5	3	3	3	pDEFG
6	2	2	3	pABCDE
6	2	3	2	pABCDF
6	3	2	2	pABCEF
6	2	3	3	pABCDG, pABDEF
6	3	2	3	pABCEG, pACDEF

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6	3	3	2	pABCFG, pBCDEF
6	4	3	2	pBCEFG
6	4	2	3	pACEFG
6	3	3	3	pABEFG, pACDFG, pBCDEG
6	3	4	2	pBCDFG
6	2	4	3	pABDFG
6	3	2	4	pACDEG
6	2	3	4	pABDEG
6	4	3	3	pCDEFG
6	3	4	3	pBDEFG
6	3	3	4	pADEFG
7	4	4	3	pBCDEFG
7	4	3	4	pACDEFG
7	3	4	4	pABDEFG
7	4	3	3	pABCEFG
7	3	4	3	pABCDFG
7	3	3	4	pABCDEG
7	3	3	3	pABCDEF
8	4	4	4	pABCDEFG

[0083] The decoding matrix provided in Table 4 is constructed using two assumptions. First, the decoding matrix assumes that the positive control (p) always produces a positive outcome. Second, the decoding matrix assumes that, within each color, the intensity is additive and scales in the same way with changing probe concentration, regardless of which probe the signal may come from. This essentially means that the signals are additive and digital. The conditions underlying these assumptions may be met by properly preparing the assay. If a fluorescent signal is used, the intensity need only be approximately additive and digital, as demonstrated in the Examples provided herein.

[0084] Table 4 allows the conversion of a cumulative measurement of intensity in four ranges of fluorescent wavelengths (i.e., signal intensity within each color range), into the corresponding analytes present in the sample. For example, a result of 4321 indicates that p, C, F, and G are present and the other analytes are not present. This result is referred to as a "legitimate" result, because it is present in the decoding matrix. By contrast, a result of 4000, while possible to measure, does not occur in the decoding matrix. More specifically, the result of 4000 cannot be achieved by adding any combination of control and analyte codes from Table 3. This result is

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referred to as an "illegitimate" result. An illegitimate result may indicate that the assay malfunctioned. Thus, the control (p) and the decoding matrix provide a means of verifying that the assay is functioning properly.

[0085] Table 4 is exhaustive for any combination of fluorophores generating four resolvable emission spectra (e.g., colors). The term "rank" is used to describe the number of detected analytes, including the control. In the example provided above, if the assay functions properly, the rank is equal to the value of the blue signal. For example, a rank of 8 indicates that the control and all seven other analytes (A-G) are present. A rank of 2 indicates that the control and only one analyte are present. The lowest rank is a rank of 1, which indicates that only the control is present. The number of possibilities at each rank can be enumerated. For example, continuing to refer to the encoding and decoding method described in Tables 3-4, there are 7 possibilities for rank 2. The number of possibilities at rank 3 can be calculated as a combination of 7 take 2, or 7!/(5! * 2!) = 21 possibilities. More generally, the number of possibilities for a combination of N take K is N!/((N-K)! * K!). Analogously, at ranks 4, 5, 6, 7, and 8, the number of possibilities is 35, 35, 21, 7, and 1, respectively. Referring to Table 4 shows that the table agrees with the theoretical prediction. Thus, Table 4 is an exhaustive decoding matrix for the encoding method provided in Table 3.

C. Reducing or Eliminating Degeneracy

[0086] The terms "degenerate" and "degeneracy," as used herein, generally describe a situation where a legitimate result is not definitive, because it can indicate more than one possibility in terms of the presence or absence of an analyte. For example, with reference to Table 4, result 5233 is degenerate because it can be decoded as either pADFG or pBDEG. Similarly, result 4222 is degenerate because it can be decoded as any of the following: pCDG, pAFG, pBEG, pDEF. By contrast, result 3110 can only indicate pBC and thus is not degenerate.

[0087] This disclosure provides methods of reducing or eliminating degeneracy, thereby increasing the confidence with which an analyte is detected. In one embodiment, degeneracy is eliminated by a method comprising (i) encoding each potential analyte to be detected as a value of a signal and, optionally, as a value in each of at least two components of a signal; (ii) enumerating every legitimate result that can be obtained from the coding scheme; (iii)

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identifying each legitimate result that is degenerate; and (iv) eliminating at least one potential analyte (or potential analyte code) from the coding scheme, wherein eliminating the at least one potential analyte reduces or eliminates degeneracy. For example, with reference to the coding scheme described in Table 3 and the decoding matrix described in Table 4 (enumerating every legitimate result), eliminating any two of analytes D, E, and F eliminates the degeneracy. Eliminating any one of analytes D, E, and F would not eliminate the degeneracy, but would reduce it.

[0088] With continued reference to the coding scheme described in Table 3, eliminating any two of analytes D, E, and F from the coding scheme results in a scheme where six analytes (including control) can be analyzed, with no degeneracy, using only 4 colors. By contrast, conventional methods of multiplexing would allow for only the reporting of 3 analytes and 1 control using 4 colors. Therefore, the number of analytes that can be analyzed is nearly doubled by using the methods provided herein.

[0089] **Fig. 2** shows an exemplary embodiment of the invention in which 4 colors are used to detect five analytes and a control. The analytes are nucleic acids from dengue virus ("Dengue Fever"), *Mycobacterium tuberculosis* ("Tuberculosis"), human immunodeficiency virus (HIV) p17 (P17), *Plasmodium* ("Malaria"), and herpes simplex ("Herpes"). The "colors" in this example are fluorophores attached to oligonucleotide probes that also comprise a quencher. The oligonucleotide probes will generally be different for different analytes, whereas the color is the same. For example, all probes designated as Probe 1 have a blue color but generally will have a different oligonucleotide sequence. Of course, probes designated as Probe 1 could also have the same oligonucleotides sequence, if the complementary sequence was present in each of the analytes. The mechanism of detection with these probes is described elsewhere in this disclosure. Probe 1 (blue) hybridizes to all six analytes, including control. Probe 2 (green) hybridizes to analytes from dengue virus, *Mycobacterium*, and HIV p17. Probe 3 (yellow) hybridizes to analytes from dengue fever and *Plasmodium*. Probe 4 hybridizes to analytes from dengue virus, *Mycobacterium*, and herpes simplex. The coding scheme defined by these probes is illustrated in Table 5. With reference to Table 3, potential analytes D (1011) and F (1110) have been eliminated from the coding scheme. Therefore, the coding scheme presented in Table

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5 is non-degenerate and each legitimate result from the assay corresponds to the presence or absence of a unique combination of analytes in a sample.

Table 5. Coding scheme for detection of dengue virus, *Mycobacterium*, HIV, *Plasmodium*, and herpes simplex, as exemplified in Fig. 2.

	Blue	**Green**	**Yellow**	**Red**
Ctrl. (p)	1	0	0	0
Herpes Simplex	1	0	0	1
Plasmodium	1	0	1	0
HIV	1	1	0	0
Mycobacterium	1	1	0	1
Dengue Virus	1	1	1	1

[0090] The methods for encoding and decoding presented above, including the methods for eliminating degeneracy, are all equally applicable to encoding methods using additional colors. For example, Table 3 could be extended by including additional colors and additional intensities (described further below). The decoding matrix is then generated as described above, enumerating every legitimate result that can be obtained from the coding scheme. A decoding matrix analogous to the decoding matrix provided in Table 4 may be constructed for any coding scheme described in this disclosure. The legitimate results that are degenerate are then identified and at least one potential analyte code is eliminated from the coding scheme to reduce or eliminate degeneracy. The method of eliminating degeneracy may be carried out using software on a computer readable medium, or hardware configured to carry out the method (e.g., a microchip).

[0091] Although degeneracy can be reduced or eliminated by the methods described above, and elsewhere in this disclosure, this disclosure also provides coding schemes that are non-degenerate by design. For example, after elimination of degeneracy in the coding scheme described in Table 3, the coding scheme may be extended indefinitely in a non-degenerate manner where the non-degeneracy is by design (see, e.g., the coding scheme exemplified in Table 6, described more fully below). Similarly, this disclosure provides coding schemes that

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are completely non-degenerate by design and therefore do not require any reduction or elimination of degeneracy (see, e.g., the coding scheme exemplified in Table 8, described more fully below). Thus, the coding schemes provided in this disclosure may have reduced or eliminated degeneracy, or be non-degenerate by design.

D. Encoding Methods Using More Than One Color and More Than One Intensity

[0092] The encoding method described above (e.g., Tables 3-5) may be further extended by allowing analytes to be encoded by an intensity greater than 1. For example, each of the analytes encoded in the coding scheme provided in Table 5 is encoded by a fluorescence intensity of either 1 or 0. Allowing higher values for the signal intensity in at least one color further increases the number of analytes that can be encoded by any of the methods provided in this disclosure. In some examples, these higher intensity values may be assigned any color except for the control color, in order to maintain the analyte counting capability of the control color.

[0093] Table 6 shows the first three tiers of an exemplary coding scheme that utilizes four colors and multiple intensities. Tier 1 of Table 6 is a reproduction of Table 3, showing the encoding of seven analytes and a control with four colors. As described above, any two of potential analytes D, E, and F may be eliminated from the coding scheme in order to produce a non-degenerate coding scheme. Tier 1 of Table 6 indicates that potential analytes D (1011) and F (1110) have been eliminated from the coding scheme to eliminate degeneracy. Therefore, the coding scheme presented in Tier 1 of Table 6 is capable of determining the presence of five analytes and one control using four colors, as described above.

[0094] The coding scheme of Tier 1 of Table 6 may be expanded to a second tier (Tier 2) by allowing the intensity in any of the colors to increase. As described above, the intensity of the control color may be maintained at 1, in order to preserve the sequence counting capability of the control. Increasing the intensity of any of the remaining three colors will yield codes 100Y, 10Y0, 1Y00, 10YY, 1Y0Y, 1YY0, and 1YYY, where $Y > 1$. The minimal value of Y for a new tier of encoding is equal to the cumulative maximum value from the prior tier(s) plus 1. As described below, a value greater than 1 could also be used, to maximize the differences between the intensities.

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[0095] Thus, in this context, the term "tier" is generally used to describe a set of codes that fully utilize the coding capability provided by a particular number of first values (e.g., intensities) and second values (e.g., colors), without degeneracy. For example, Tier 1 of Table 6 fully utilizes the coding capability provided by four colors with up to one intensity in each color, without degeneracy. As shown in Table 6, Tier 1, this results in six encoded analytes, including the control. To introduce a second tier, the minimum value of Y (described above) may be incremented to equal the cumulative maximum result from the prior tier(s) plus one (or more than one). In the example provided in Table 6, the intensity of the blue (control) color is maintained as one, to preserve the sequence counting capability in this color. Thus, Tier 2 consists of five non-degenerate encoding possibilities obtained by incrementing the intensities in the green, yellow, and red channels to equal the cumulative maximum results in each of these channels from Tier 1, plus one. All possibilities of these codes may then be enumerated for Tier 2, and codes resulting in degeneracy may be eliminated, or Tier 2 may be made non-degenerate by design, using the information used to eliminate the corresponding codes from Tier 1. Further coding capacity may then be achieved by adding a third tier, or further tiers, which are constructed according to analogous methods. A coding scheme may have an infinite number of tiers, for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100 or more tiers.

[0096] More specifically, with reference to Table 6, Tier 2, analyte H is encoded by 1004. The value of the control color is maintained as 1. The value of the red color is equal to the cumulative maximum result from the prior tier (3) plus 1, or 4. Similarly, analytes I and J are encoded by 1030 and 1400, respectively. Combinations of these codes are used to encode the remaining four analytes (K-N), as was done for analytes D-G in Tier 1. This completes Tier 2. In Tier 2, the inclusion of potential analytes K (1034) and M (1430) results in degeneracy. Therefore, these analytes have been eliminated from the coding scheme, to eliminate degeneracy.

[0097] Continuing to refer to Table 6, a third tier (Tier 3) is constructed using the same principles described above. Analyte O is encoded by 1-0-0-16. The value of the control color is still maintained as 1. The value of the red color is equal to the cumulative maximum result from the prior tiers (15) plus 1, or 16. Similarly, analytes P and Q are encoded by 1-0-9-0 and 1-16-0-

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0, respectively. Combinations of these codes are used to encode the remaining four analytes (R-U), as was done for analytes D-G in Tier 1 and analytes K-N in Tier 2. In Tier 3, the inclusion of potential analytes R (1-0-9-16) and T (1-16-9-0) results in degeneracy. Therefore, these analytes have been eliminated from the coding scheme, to eliminate degeneracy.

[0098] The three-tier coding scheme shown in Table 6 shows the encoding of 15 analytes and one control using four colors. This coding scheme may be indefinitely extended, by adding more intensities to generate additional tiers and/or adding more colors, to generate additional coding capability within the tiers. The methods of reducing or eliminating degeneracy, as described in this disclosure, may be used with the addition of each intensity and/or color, to reduce or eliminate degenerate results.

[0099] More generally, the coding scheme depicted in Table 6 is a non-degenerate, infinite extension of the coding scheme depicted in Tables 3-5. The maximum intensity of the cumulative measurement at the first tier is 6. The maximum intensity of the cumulative measurement at the second tier is 15. The maximum intensity of the cumulative measurement at the third tier is 63, and so on. Given a maximum cumulative intensity value (F), the maximum number of tiers (T) available in this coding scheme is $T = \log_4 (F + 1)$. The coding scheme depicted in Table 6 provides five non-degenerate codes per tier (P). Thus, the maximum number of codes $M = 5 * \log_4 (F + 1)$, or $M = P * T$, where P is the number of non-degenerate codes per tier and T is the number of tiers. For example, given $F = 63$, the maximum number of codes (i.e., analytes) is 15 for 5 non-degenerate codes per tier. This formula does not include the 1000 code, which is reserved for a positive control in Table 6. To include the control in the total number of analytes, one would simply add one, to provide the formula $M = (P * T) + 1$.

[00100] The methods provided in this disclosure may be used to expand this coding scheme infinitely. For example, by utilizing combinations of different intensities (i.e., first values) and colors (i.e., second values) one can encode any number of analytes (M) by varying the number of non-degenerate codes per tier (P) and the number of tiers (T). For example, the number of non-degenerate codes per tier is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more. Similarly, the number of tiers may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29,

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30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more.

Table 6. Encoding of 15 analytes and one control using four colors and multiple intensities.

Tier	Analyte	B	G	Y	R	Comments
1	**Ctrl. (p)**	1	0	0	0	
	A	1	0	0	1	
	B	1	0	1	0	
	C	1	1	0	0	
	~~**D**~~	~~1~~	~~0~~	~~1~~	~~1~~	Eliminated from coding scheme to eliminate degeneracy.
	E	1	1	0	1	
	~~**F**~~	~~1~~	~~1~~	~~1~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	G	1	1	1	1	
Cumulative Maximum Result		*6*	*3*	*2*	*3*	
2	**H**	1	0	0	4	
	I	1	0	3	0	
	J	1	4	0	0	
	~~**K**~~	~~1~~	~~0~~	~~3~~	~~4~~	Eliminated from coding scheme to eliminate degeneracy.
	L	1	4	0	4	
	~~**M**~~	~~1~~	~~4~~	~~3~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	N	1	4	3	4	
Cumulative Maximum Result		*11*	*15*	*8*	*15*	
3	**O**	1	0	0	16	
	P	1	0	9	0	
	Q	1	16	0	0	
	~~**R**~~	~~1~~	~~0~~	~~9~~	~~16~~	Eliminated from coding scheme to eliminate degeneracy.
	S	1	16	0	16	
	~~**T**~~	~~1~~	~~16~~	~~9~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	U	1	16	9	16	
Cumulative						

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Maximum Result						
Cumulative Maximum Result for Three Non-Degenerate Tiers		***16***	***63***	***26***	***63***	

[00101] The right-hand side of **Fig. 1** shows one exemplary embodiment of the method described above. More specifically, the right-hand side of **Fig. 1** shows an encoding method where nine or more analytes (Seq 1 - Seq 9, etc.) are each encoded by at least two colors, with varying intensities within each color. The color represents a fluorophore attached to an oligonucleotide probe that also comprises a quencher. The system can be designed so that each probe is labeled with single fluorophore, or each probe is labeled with more than one fluorophore. For example, the code for analyte H, in Table 6, is 1004. The intensity of 4 in the red channel may be achieved by either using an H-specific probe comprising 4 red fluorophores, or by using 4 H-specific probes each comprising a single red fluorophore. Of course, any combination of probes and fluorophores producing a result of 4 in the red channel would be equally appropriate, such as 2 probes with 2 red fluorophores each, and 1 probe with 1 red fluorophore and 1 probe with 3 red fluorophores, or simply one probe with one red fluorophore but present at 4x amount in the reaction mixture.

[00102] The coding scheme depicted on the right-hand side of **Fig. 1** may be represented as in Table 7. The result of the analysis shown on the right-hand side of **Fig. 1** is 4112, or an intensity of 4 in the blue channel, 1 in the green channel, 1 in the yellow channel, and 2 in the red channel. Using a decoding matrix constructed as described herein, this result is decoded to indicate the presence of Seq 1, Seq 3, Seq 4, and Seq 5.

Table 7. Coding scheme illustrated on the right-hand side of Fig. 1.

Tier	Analyte	B	G	Y	R	Comments
1	**Seq 1**	1	0	0	0	
	Seq 2	1	1	0	0	

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	Seq 3	1	0	1	0	
	Seq 4	1	0	0	1	
	Seq 5	1	1	0	1	
	Seq 6	1	1	1	1	
	~~**Eliminated Potential Seq**~~	~~1~~	~~0~~	~~1~~	~~1~~	Eliminated from coding scheme to eliminate degeneracy.
	~~**Eliminated Potential Seq**~~	~~1~~	~~1~~	~~1~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
Cumulative Maximum Result		*6*	*3*	*2*	*3*	
2	**Seq 7**	1	4	0	0	
	Seq 8	1	0	3	0	
	Seq 9	1	0	0	4	
Etc. - i.e. Continue as provided in Table 6.						

E. Encoding Methods Using One Color and One Intensity Per Analyte but Different Intensities Among Analytes

[00103] In some methods provided herein, each analyte is encoded by a single color and intensity combination. For example, in a four color system, the first four analytes may be encoded by 1000, 2000, 4000, and 8000. The next four analytes may be encoded by 0100, 0200, 0400, and 0800. Analytes 9-12 and 13-16 would be assigned analogously, as shown in Table 8.

[00104] Like the encoding method described in Table 6, this coding scheme is theoretically infinite, non-degenerate by construction, and only limited by the bandwidth of the instrument used to measure the signal. However, this coding scheme enables more analytes to be quantified per unit of bandwidth than the encoding method described in Table 6. The reason is that the available multiplicity of signal is used with maximal efficiency, as each level of intensity is utilized in the coding (i.e. there are no gaps in the chromatogram; see below for description of chromatograms). Table 8 shows one embodiment of this method, illustrating four tiers of encoding based on four colors and intensities 1, 2, 4, and 8. The coding scheme is non-degenerate, and the result if all 16 analytes are present is 15-15-15-15. This encoding method is more efficient, in terms of bandwidth utilization, than the encoding methods presented above.

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However, this method does not have the proofreading capability of the first scheme, as all the results decode to legitimate outcomes in the absence of gaps in the chromatogram.

Table 8. Example of encoding method using one color and one intensity per analyte, but different intensities among analytes.

Tier	Analyte	B	G	Y	R
1	**A**	1	0	0	0
	B	2	0	0	0
	C	4	0	0	0
	D	8	0	0	0
2	**E**	0	1	0	0
	F	0	2	0	0
	G	0	4	0	0
	H	0	8	0	0
3	**I**	0	0	1	0
	J	0	0	2	0
	K	0	0	4	0
	L	0	0	8	0
4	**M**	0	0	0	1
	N	0	0	0	2
	O	0	0	0	4
	P	0	0	0	8
		15	***15***	***15***	***15***

[00105] The method presented above, and exemplified in Table 8, may be extended by introducing additional colors and/or intensities. For example, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more colors may be used. Additional intensity levels, such as 16, 32, 64, 128, 255, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, and so on may also be used. The method is generalizable and may be used to encode an infinite number of analytes. Each analyte is represented by a code in a single color, wherein the value of

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the code in that single color equal to the sum of all previous values plus one. For example, if the first code contains a 1 in a particular color, the next codes are 2, 4, 8, 16, 32, 64, 128 and so on. Other progressions are possible, but will not be as efficient in terms of usage of the bandwidth. However, one of ordinary skill in the art will recognize that less efficient use of bandwidth may be desirable when one wishes to maximize separation between values. Similarly, the value 1 could be excluded from the coding scheme, for example to maximize the difference in the intensity between the first encoded value and instrumental noise. For example, in some cases a coding scheme may begin with a value of 2, which would provide a progression of 2, 4, 8, 16, 32, 64, 128, and so on for the analyte codes, while the progression of cumulative results would be 2, 4, 6, 8, 10, 12, 14, 16, and so on. This approach generates uniform gaps in the possible cumulative results, allowing for higher tolerance to noise in comparison to an analyte code progression of 1, 2, 4, 8, 16, 32, and so on, and its corresponding cumulative result progression of 1, 2, 3, 4, 5, 6, 7, 8, and so on. In this example, the increased robustness in the measurement comes at a cost of a decrease in the number of available codes within a fixed bandwidth of multiplicity. For example, if only 63 multiplicity states can be measured reliably, the coding scheme starting with 1 offers 7 codes per color whereas the coding scheme starting with 2 offers 6 codes per color. If 4 colors are available, the former will offer 28 codes, while the latter will offer 24 codes. In summary, both the starting signal intensity and the progression (i.e., difference between intensity values) may be scaled in order to maximize the ability to distinguish over instrumental noise and maximize the differences between the intensity values, thereby enhancing the ability to distinguish between distinct experimental outcomes.

[00106] The coding scheme illustrated in Table 8 is non-degenerate by design. Although the coding scheme illustrated in Table 8 uses both intensity and color to encode each of the 16 analytes, each of the analytes could also be encoded by simply utilizing intensity. For example, given the 16 analytes provided in Table 8 (A-P), a single color coding scheme encoding all 16 analytes may assign the values 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, and 16384 to analytes A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, and P, respectively. Such a coding scheme is non-degenerate by design and the cumulative intensity result can be unambiguously decoded to indicate the presence or absence of analytes A-P. This coding scheme is capable of detecting the presence or absence of M analytes, where $M = \log_2 (F + 1)$ and F is the maximum

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cumulative value of a signal (e.g., signal intensity). By adding a second component to this signal (e.g., color, as depicted in Table 8), the capacity of this coding scheme can be increased to $M = C * \log_2 (F + 1)$, where C is the number of colors used in said coding scheme.

[00107] As described throughout this specification, any suitable value may be used for C or F. For example, C may be at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more. F may be at least about 2, 4, 8, 16, 32, 64, 128, 255, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, 131072, 262144, 524288, 1048576, and so on. As described elsewhere in this disclosure, both the initial starting value of F and the steps in its progression may also be varied.

III. Analytes

[00108] An analyte may be any suitable analyte that may be analyzed using the methods and compositions of the invention, where the analyte is capable of interacting with a reagent in order to generate a signal with at least two components that can be measured. An analyte may be naturally-occurring or synthetic. An analyte may be present in a sample obtained using any methods known in the art. In some cases, a sample may be processed before analyzing it for an analyte. The methods and compositions presented in this disclosure may be used in solution phase assays, without the need for particles (such as beads) or a solid support.

[00109] In some cases, an analyte may be a polynucleotide, such as DNA, RNA, peptide nucleic acids, and any hybrid thereof, where the polynucleotide contains any combination of deoxyribo- and/or ribo-nucleotides. Polynucleotides may be single stranded or double stranded, or contain portions of both double stranded or single stranded sequence. Polynucleotides may contain any combination of nucleotides or bases, including, for example, uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine, isoguanine and any nucleotide derivative thereof. As used herein, the term "nucleotide" may include nucleotides and nucleosides, as well as nucleoside and nucleotide analogs, and modified nucleotides, including both synthetic and naturally occurring species. Polynucleotides may be any suitable polynucleotide for which one or more reagents (or probes) as described herein may be produced, including but not limited to cDNA, mitochondrial DNA (mtDNA), messenger RNA (mRNA),

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ribosomal RNA (rRNA), transfer RNA (tRNA), nuclear RNA (nRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small Cajal body-specific RNA (scaRNA), microRNA (miRNA), double stranded (dsRNA), ribozyme, riboswitch or viral RNA. Polynucleotides may be contained within any suitable vector, such as a plasmid, cosmid, fragment, chromosome, or genome.

[00110] Genomic DNA may be obtained from naturally occurring or genetically modified organisms or from artificially or synthetically created genomes. Analytes comprising genomic DNA may be obtained from any source and using any methods known in the art. For example, genomic DNA may be isolated with or without amplification. Amplification may include PCR amplification, multiple displacement amplification (MDA), rolling circle amplification and other amplification methods. Genomic DNA may also be obtained by cloning or recombinant methods, such as those involving plasmids and artificial chromosomes or other conventional methods (see Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*., cited *supra*.) Polynucleotides may be isolated using other methods known in the art, for example as disclosed in *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV) or *Molecular Cloning: A Laboratory Manual*. If the isolated polynucleotide is an mRNA, it may be reverse transcribed into cDNA using conventional techniques, as described in Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*., cited *supra*.

[00111] An analyte may be a protein, polypeptide, lipid, carbohydrate, sugar, small molecule, or any other suitable molecule that can be detected with the methods and compositions provided herein. An analyte may be an enzyme or other protein. An analyte may be a drug or metabolite (e.g., anti-cancer drug, chemotherapeutic drug, anti-viral drug, antibiotic drug, or biologic). An analyte may be any molecule, such as a co-factor, receptor, receptor ligand, hormone, cytokine, blood factor, antigen, steroid, or antibody.

[00112] An analyte may be any molecule from any pathogen, such as a virus, bacteria, parasite, fungus, or prion (e.g., PrP^{Sc}). Examples of viruses include those from the families Adenoviridae, Flaviviridae, Hepadnaviridae, Herpesviridae, Orthomyxoviridae, Papovaviridae, Paramyxoviridae, Picornaviridae, Polyomavirus, Retroviridae, Rhabdoviridae, and Togaviridae. Specific examples of viruses include adenovirus, astrovirus, bocavirus, BK virus, coxsackievirus, cytomegalovirus, dengue virus, Ebola virus, enterovirus, Epstein-Barr virus, feline leukemia

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virus, hepatitis virus, hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis D virus, hepatitis E virus, herpes simplex virus (HSV), HSV type 1, HSV type 2, human immunodeficiency virus (HIV), HIV type 1, HIV type 2, human papilloma virus (HPV), HPV type 1, HPV type 2, HPV type 3, HPV type 4, HPV type 6, HPV type 10, HPV type 11, HPV type 16, HPV type 18, HPV type 26, HPV type 27, HPV type 28, HPV type 29, HPV type 30, HPV type 31, HPV type 33, HPV type 34, HPV type 35, HPV type 39, HPV type 40, HPV type 41, HPV type 42, HPV type 43, HPV type 44, HPV type 45, HPV type 49, HPV type 51, HPV type 52, HPV type 54, HPV type 55, HPV type 56, HPV type 57, HPV type 58, HPV type 59, HPV type 68, HPV type 69, influenza virus, JC virus, Marburg virus, measles virus, mumps virus, Norwalk virus, parovirus, polio virus, rabies virus, respiratory syncytial virus, retrovirus, rhinovirus, rotavirus, Rubella virus, smallpox virus, vaccinia virus, West Nile virus, and yellow fever virus.

[00113] Examples of bacteria include those from the genuses *Bordetella, Borrelia, Brucella, Campylobacter*, *Chlamydia, Clostridium, Corynebacterium, Enterococcus, Escherichia, Francisella, Haemophilus, Helicobacter, Legionella, Leptospira, Listeria, Mycobacterium*, *Mycoplasma, Neisseria, Pseudomonas*, *Rickettsia, Salmonella*, *Shigella*, *Staphylococcus, Streptococcus*, *Treponema, Vibrio, and Yersinia.* Specific examples of bacteria include *Bordetella par apertussis, Bordetella pertussis, Borrelia burgdorferi, Brucella abortus, Brucella canis, Brucella melitensis, Brucella suis, Campylobacter jejuni, Chlamydia pneumoniae, Chlamydia psittaci, Chlamydia trachomatix, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium tetani, Corynebacterium diphtheriae, Enterococcus faecalis, Enterococcus faecium, Escherichia coli*, *Francisella tularensis, Haemophilus influenzae, Helicobacter pylori, Legionella pneumophila, Leptospira interrogans, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Mycobacterium ulcerans, Mycoplasma pneumoniae, Neisseria gonorrhoeae, Neisseria meningitidis, Pseudomonas aeruginosa, Rickettsia rickettsii, Salmonella choleraesuis, Salmonella dublin, Salmonella enteritidis, Salmonella typhi, Salmonella typhimurium, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus agalactiae, Streptococcus pneumoniae, Streptococcus pyogenes, Treponema pallidum, Vibrio cholerae, Yersinia pestis, and Yersinia enterocolitica.*

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[00114] Examples of parasites include those from the genuses *Acanthamoeba, Babesia, Balamuthia, Balantidium, Blasocystis, Cryptosporidium, Dientamoeba, Entamoeba, Giardia, Isospora, Leishmania, Naegleria, Pediculus, Plasmodium, Rhinosporidium, Sarcocystis, Schistosoma, Toxoplasma, Trichomonas,* and *Trypanosoma.* Specific examples of parasites include *Babesia divergens, Babesia bigemina, Babesia equi, Babesia microfti, Babesia duncani, Balamuthia mandrillaris, Balantidium coli, Dientamoeba fragilis, Entamoeba histolytica, Giardia lamblia, Isospora belli, Naegleria fowleri, Pediculus humanus, Plasmodium falciparum, Plasmodium knowlesi, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, Rhinosporidium seeberi, Sarcocystis bovihominis, Sarcocystis suihominis, Schistosoma mansoni, Toxoplasma gondii, Trichomonas vaginalis, Trypanosoma brucei,* and *Trypansoma cruzi.*

[00115] Examples of fungi include those from the genuses *Apophysomyces, Aspergillus, Blastomyces, Candida, Cladosporium, Coddidioides, Cryptococcos, Exserohilum, Fusarium, Histoplasma, Pichia, Pneumocystis, Saccharomyces, Sporothrix, Stachybotrys,* and *Trichophyton.* Specific examples of fungi include *Aspergillus fumigatus, Aspergillus flavus, Aspergillus clavatus, Blastomyces dermatitidis, Candida albicans, Coccidioides immitis, Crytptococcus neoformans, Exserohilum rostratum, Fusarium verticillioides, Histoplasma capsulatum, Pneumocystis jirovecii, Sporothrix schenckii, Stachybotrys chartarum,* and *Trichophyton mentagrophytes.*

[00116] In some cases, the methods provided in this disclosure may be used to detect any one of the analytes described above, or elsewhere in the specification. In some cases the methods provided in this disclosure may be used to detect panels of the analytes described above, or elsewhere in the specification. For example, a panel may comprise an analyte selected from the group consisting of any 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100 or more analytes described above or elsewhere in the specification.

[00117] An analyte may be obtained from any suitable location, including from organisms, whole cells, cell preparations and cell-free compositions from any organism, tissue, cell, or environment. Analytes may be obtained from environmental samples, biopsies, aspirates, formalin fixed embedded tissues, air, agricultural samples, soil samples, petroleum samples, water samples, or dust samples. In some instances, an analyte may be obtained from bodily

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fluids which may include blood, urine, feces, serum, lymph, saliva, mucosal secretions, perspiration, central nervous system fluid, vaginal fluid, or semen. Analytes may also be obtained from manufactured products, such as cosmetics, foods, personal care products, and the like. Analytes may be the products of experimental manipulation including, recombinant cloning, polynucleotide amplification, polymerase chain reaction (PCR) amplification, purification methods (such as purification of genomic DNA or RNA), and synthesis reactions.

[00118] More than one type of analyte may be detected in each multiplexed assay. For example, a polynucleotide, a protein, a polypeptide, a lipid, a carbohydrate, a sugar, a small molecule, or any other suitable molecule may be detected simultaneously in the same multiplexed assay with the use of suitable reagents. Any combination of analytes may be detected at the same time.

[00119] Detection of an analyte may be useful for any suitable application, including research, clinical, diagnostic, prognostic, forensic, and monitoring applications. Exemplary applications include detection of hereditary diseases, identification of genetic fingerprints, diagnosis of infectious diseases, cloning of genes, paternity testing, criminal identification, phylogeny, anti-bioterrorism, environmental surveillance, and DNA computing. For example, an analyte may be indicative of a disease or condition. An analyte may be used to make a treatment decision, or to assess the state of a disease. The presence of an analyte may indicate an infection with a particular pathogen, or any other disease, such as cancer, autoimmune disease, cardiorespiratory disease, liver disease, digestive disease, and so on. The methods provided herein may thus be used to make a diagnosis and to make a clinical decision based on that diagnosis. For example, a result that indicates the presence of a bacterial polynucleotide in a sample taken from a subject may lead to the treatment of the subject with an antibiotic.

[00120] In some cases the methods and compositions of the invention may be used to detect at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes. In some cases the methods and compositions of the invention may be used to detect 7-50, 8-40, 9-30, 10-20, 10-15, 8-12, or 7-12 analytes.

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[00121] In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis.

IV. Signals

[00122] The methods presented in this disclosure may be used with any quantifiable signal. In some cases, this disclosure provides coding schemes and methods to encode an infinite number of targets without degeneracy, using a single component of a signal (e.g., intensity). For example, as described above and in Example 3, a coding scheme may rely on a multiplicity of signal intensity without consideration of color. Although fluorescent probes have been used to illustrate this principle, the coding scheme is equally applicable to any other method providing a quantifiable signal, including an electrochemical signal and a chemiluminescent signal, as described elsewhere in this disclosure.

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[00123] The methods presented in this disclosure may also utilize the measurement of a signal in at least two dimensions, also referred to as the measurement of at least two components of a signal. In comparison to the coding scheme described in the paragraph above, which relies on, for example, signal intensity to differentiate between analytes, utilization of at least two components of a signal (e.g., color and intensity) allows the generation of more unique codes per unit of signal intensity bandwidth. When at least two components of a signal are utilized, a cumulative measurement of the at least two components may be obtained for a single sample volume. For example in the coding scheme described in Table 5, the presence of each analyte results in a particular intensity (one component of the signal) in each of the four colors (a second component of a signal). The combination of these constituent signals leads to a cumulative signal that may be measured by measuring an intensity at each wavelength or range of wavelengths. The corresponding coding scheme or decoding matrix may then be used to convert the cumulative measurement into a determination of the presence or absence of an analyte.

[00124] In some cases, a quantifiable signal comprises a waveform that has both a frequency (wavelength) and an amplitude (intensity). A signal may be an electromagnetic signal. An electromagnetic signal may be a sound, a radio signal, a microwave signal, an infrared signal, a visible light signal, an ultraviolet light signal, an x-ray signal, or a gamma-ray signal. In some cases, an electromagnetic signal may be a fluorescent signal, for example a fluorescence emission spectrum that may be characterized in terms of wavelength and intensity.

[00125] In certain portions of this disclosure, the signal is described and exemplified in terms of a fluorescent signal. This is not meant to be limiting, and one of ordinary skill in the art will readily recognize that the principles applicable to the measurement of a fluorescent signal are also applicable to other signals. For example, like fluorescent signals, any of the electromagnetic signals described above may also be characterized in terms of a wavelength and an intensity. The wavelength of a fluorescent signal may also be described in terms of color. The color may be determined based on measuring intensity at a particular wavelength or range of wavelengths, for example by determining a distribution of fluorescent intensity at different wavelengths and/or by utilizing a band pass filter to determine the fluorescence intensity within a particular range of wavelengths. Such band pass filters are commonly employed in a variety of

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laboratory instrumentation, including quantitative PCR machines. Intensity may be measured with a photodetector. A range of wavelengths may be referred to as a "channel."

[00126] In some cases, the methods provided in this disclosure may be used with any signal where the cumulative signal scales with the constituent signals of the same color, frequency, absorption band, and so on. However, the cumulative signal need not be digital or scale linearly with the number and intensity of the constituent signals. For example, if the physical principle of measurement is absorption, the cumulative attenuation is a product of constituent attenuations while the constituent concentrations are additive, due to the exponential nature of the Beer-Lambert law. The logarithm of the cumulative attenuation will then scale linearly with constituent concentrations in each absorption band (the equivalent of color, if fluorescent detection is used). The methods of the invention are therefore applicable. The methods of the invention may also be used with chemiluminescent signals and electrochemical signals.

[00127] The number of signals, and the number of dimensions or components measured, may also be expanded beyond the numbers shown in the exemplary embodiments of the invention, leading to an expansion in multiplexing capability. The exemplary embodiments provided in this disclosure utilize coding schemes constructed utilizing a fluorescent signal with one or two components measured: wavelength and/or intensity. The number of analytes that can be encoded can be increased by increasing the number of wavelengths and/or intensities. The number of analytes that can be encoded can also be increased by increasing the number of signals, for example by combining a fluorescent signal with an electrochemical signal or a FRET signal (fluorescence resonance energy transfer).

[00128] In some cases, more than two components of a signal may be measured. For example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, 20 or more components of a signal may be measured. At least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, or 20 components of a signal may be measured. At least 2, but fewer than 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, or 20 components of a signal may be measured. In some cases, 2-3, 2-4, 2-5, 2-6, 3-5, 3-6, 3-8, or 5-10 components of a signal may be measured. These additional components may include kinetic components, such as a rate of signal decay and rate of photobleaching.

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[00129] If a fluorescent signal is employed, the number of analytes that can be encoded may be further expanded by utilizing additional fluorophores. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores may be used. In some cases, fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores may be used. In some cases, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 fluorophores may be used.

[00130] Generally, the number of analytes that can be encoded may be further expanded by utilizing additional first values (e.g., intensities) that are values or ranges of values from a first component of a signal. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more first values that are values or ranges of values from a first component of a signal may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more first values that are values or ranges of values from a first component of a signal may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 first values that are values or ranges of values from a first component of a signal may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 first values that are values or ranges of values from a first component of a signal may be used.

[00131] In instances in which intensity is a component of a signal that is quantified, such as when a fluorescent signal is utilized, the presence of an analyte may be encoded using a variety of intensities or ranges of intensities. For example, a coding scheme may utilize 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more intensities or ranges of intensities. A coding scheme may utilize at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,

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14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more intensities or ranges of intensities. A coding scheme may utilize fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 intensities or ranges of intensities. In some cases, a coding scheme may utilize 2-20, 2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9, 2-10, 3-4, 3-5, 3-6, 3-7, 3-8, 3-9, 3-10, 4-5, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, or 5-10 intensities or ranges of intensities.

[00132] The number of analytes that can be encoded may be further expanded by utilizing additional second values (e.g., wavelengths) that are values or ranges of values from a second component of a signal. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second values that are values or ranges of values from a second component of a signal may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more second values that are values or ranges of values from a second component of a signal may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 second values that are values or ranges of values from a second component of a signal may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 second values that are values or ranges of values from a second component of a signal may be used.

[00133] In instances in which wavelength is a component of a signal that is quantified, such as when a fluorescent signal is utilized, the presence of an analyte may be encoded using a variety of wavelengths or ranges of wavelengths. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more wavelengths or ranges of wavelengths may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more wavelengths or ranges of wavelengths may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27,

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28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 wavelengths or ranges of wavelengths may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 wavelengths or ranges of wavelengths may be used.

[00134] In some cases, when degeneracy is eliminated, the methods of the invention are capable of detecting the presence or absence of at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more analytes in a single volume when each reagent used to generate a signal in the volume generates only one second value (e.g., each reagent, emits light at only one wavelength).

[00135] In other cases, when degeneracy is eliminated, the methods of the invention are capable of detecting the presence or absence of at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more analytes in a single volume using a total of four second values in that volume (e.g., a total of four wavelengths or ranges or wavelengths, which might be implemented by using four spectrally resolvable fluorophores).

[00136] As described throughout the specification, the assay provided herein utilizes cumulative measurements on a sample. A cumulative measurement may be, for example, a single measurement of intensity values, or a measurement of intensity values at one or more wavelengths or ranges of wavelengths. A plurality of cumulative measurements may be obtained. For example, an intensity may be measured at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more wavelengths or ranges of wavelengths. An intensity may be measured at at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more wavelengths or ranges of wavelengths. An intensity may be measured at less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 wavelengths or ranges of wavelengths.

[00137] More generally a cumulative measurement may be obtained for any quantifiable component of a signal, and for any quantifiable component of a signal at another quantifiable

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component of a signal. For example, at least a first component of a signal may be measured at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second components of a signal. At lest a first component of a signal may be measured at at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second components of a signal. At least a first component of a signal may be measured at less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 second components of a signal.

[00138] As is apparent from this disclosure, each analyte to be detected can be encoded as a code utilizing any number of suitable components of a signal or any number of signals. For example, each analyte to be detected can be encoded in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more components of a signal or signals. In some cases, each analyte to be detected can be encoded in at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more components of a signal or signals. In some cases, each analyte to be detected can be encoded in fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 components of a signal or signals. In some cases, 1-4, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 components of signal or signals may be used to encode each analyte. Each analyte in a coding scheme may be encoded by the same number of components of a signal, or signals, or different numbers of components of a signal, or signals.

A. Probes, Primers, Fluorophores, and Quenchers

[00139] Some of the methods provided in this disclosure utilize a reagent that generates a signal in the presence of an analyte. Any suitable reagent may be used with the invention. Generally, a reagent will have an analyte-specific component and a component that generates a

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signal in the presence of the analyte. In some cases, these reagents are referred to as probes. The probes may be hybridization probes. The hybridization probes may be an oligonucleotide probe attached to a fluorophore and a quencher (e.g., a TAQMAN probe).

[00140] The methods of the invention may use one or more reagents or probes to detect the presence or absence of each analyte. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more or probes may be used to detect the presence or absence of each analyte. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 probes may be used to detect the presence or absence of each analyte. In some cases, fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, , 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 probes may be used to detect the presence or absence of each analyte. In some cases, the number of probes used to detect the presence or absence of each analyte is 1-10, 2-9, 2-8, 2-7, 2-6, 2-5, 2-4, 1-3, or 1-4.

[00141] In some cases, a sample is contacted with 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more probes to detect the presence or absence of all analytes. In some cases, a sample is contacted with at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more probes to detect the presence or absence of all analytes. In some cases, a sample is contacted with fewer than 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 probes to detect the presence or absence of all analytes. In some cases, the number of probes that a sample is contacted with to detect the presence or absence of all analytes is 4-50, 4-40, 4-30, 4-20, 5-15, 5-10, or 3-10.

[00142] As described above, oligonucleotide probes attached to a fluorophore and a quencher may be used to detect the presence of an analyte in a polynucleotide amplification assay. So long as the quencher and the fluorophore are in proximity, the quencher quenches the fluorescence emitted by the fluorophore upon excitation by a light source. The sequence of the

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oligonucleotide probe is designed to be complementary to a polynucleotide sequence present in an analyte, and therefore capable of hybridizing to the polynucleotide sequence present in the analyte. Hybridization of the oligonucleotide probe may be performed in a nucleic acid amplification reaction comprising primers (e.g., a polymerase chain reaction). Upon extension of the primers by a DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and the quencher into the medium. The proximity between the fluorophore and the quencher is broken and the signal from the fluorophore is no longer quenched. Thus, the amount of fluorescence detected is a function of the amount of analyte present. If no analyte is present, the probe will not hybridize to an analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be produced.

[00143] Oligonucleotide probes may have one or a plurality of fluorophores and quenchers per probe. For example, in some embodiments an oligonucleotide probe may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores. An oligonucleotide probe may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores. An oligonucleotide probe may comprise fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores.

[00144] An oligonucleotide probe may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more quenchers. An oligonucleotide probe may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 quenchers. An oligonucleotide probe may comprise fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 quenchers.

[00145] Attachment of probes and quenchers to a probe may be performed in the same reaction or in serial reactions. A series of reactions may be performed to label probes with at

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least one fluorophore and the reaction products may be mixed to generate a mixture of probes with different fluorophores.

[00146] If an oligonucleotide probe comprises two or more fluorophores, these fluorophores may be arranged to allow Förster (Fluorescence) resonance energy transfer (FRET) to occur between the fluorophores. Briefly, FRET is a mechanism of energy transfer between fluorophores. Using FRET-based probes allows one excitation source to generate two different fluorescence emission signals by excitation of a single fluorophore. For example, the methods described herein may used paired probes, where one probe in the pair is attached to a fluorophore and a quencher and a second probe is attached to two fluorophores (in close enough proximity for FRET to occur) and a quencher. Any combination of fluorophores and quenchers that provide for FRET may be used. Such an approach doubles the number of fluorescent probes that may be used with an excitation source, because a single excitation source can be used to produce two signals: one from the probe with one fluorophore and one from the probe with two fluorophores in close enough proximity to provide for FRET. For example, using the encoding method described in Table 8, the number of unambiguously detectable analytes could be increased from 16 to 32 by pairing each of the probes in Table 8 with a corresponding FRET probe.

[00147] The primers may be specific for a particular analyte and capable of amplifying a region complementary to a probe. In some cases, the number of pairs of primers used is equivalent to the number of probes. In other cases, the number of probes used may exceed the number of primer pairs used. In still other cases, the number of primer pairs used may exceed the number of probes used. In some cases a sample to be analyzed is contacted with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more pairs of primers. In some cases a sample to be analyzed is contacted with at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more pairs of primers. In some cases a sample to be analyzed is contacted with fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44,

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45, 46, 47, 48, 49, or 50 pairs of primers. In some cases, the number of pairs of primers is 2-10, 3-15, 4-20, 3-10, 4-10, 5-10, 6-8, or 6-10.

[00148] The primers may amplify regions of a polynucleotide in which different numbers of hybridization probes hybridize. For example, at least one pair of primers may amplify a region complementary to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 hybridization probes. In some cases, all of said pairs of primers may amplify a region complementary to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 hybridization probes.

[00149] In one example, a first probe may be labeled with the fluorophore 5'-fluorescein amidite (5'-FAM) and the quencher black hole quencher 1 (BHQ-1). A second probe may be labeled with 5'-FAM in close proximity (e.g., attached to) to cyanine 5.5 (Cy5.5) and the quencher black hole quencher 3 (BHQ-3). Upon digestion of the probe, via the nuclease activity of the polymerase, two fluorescent signals are generated from a single excitation wavelength (e.g., 470 nm). The fluorophore from the first probe will fluoresce at about 520 nm. The fluorophores on the second probe undergo FRET. The donor fluorophore, FAM is excited by the excitation light source (e.g., 470 nm) and transfers its energy to the acceptor fluorophore, Cy5.5. The acceptor fluorophore emits at about 705 nm. These fluorophores and quenchers are merely exemplary. Any fluorophores that can undergo FRET and any quenchers that can quench fluorescence are suitable for use with the invention. Methods for producing FRET-based probes are described in Jothikumar et al., BioTechniques, 2009, 46(7):519-524.

[00150] In some examples, a single hybridization probe may be used for each analyte. In order to utilize multi-color encoding (e.g., as shown in Table 3), each probe may be labeled with a plurality of fluorophores at pre-determined ratios. The ratio may be determined so that a positive control signal provides the same intensity as other positive control signals of the same intensity, in the same color. This approach reduces the number of probes that must be synthesized and may be less expensive to deploy than methods utilizing multiple probes. Moreover, in the case of hybridization probes, it is easier to fit one hybridization probe to an analyte sequence than multiple probes.

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[00151] Although many aspects of the invention are exemplified using nucleic acid-based probes, one of ordinary skill in the art will readily recognize that other forms of probes would work equally well with the invention described in this disclosure. For example, a binding molecule specific to an analyte could be used as a probe. Non-limiting exemplary binding molecules include an antibody recognizing an analyte, and generating a signal in the presence of an analyte.

[00152] In embodiments of the invention that utilize fluorescent labels, any suitable fluorescent label may be used. Exemplary fluorescent labels suitable for use with the invention include rhodamine, rhodol, fluorescein, thiofluorescein, aminofluorescein, carboxyfluorescein, chlorofluorescein, methylfluorescein, sulfofluorescein, aminorhodol, carboxyrhodol, chlororhodol, methylrhodol, sulforhodol; aminorhodamine, carboxyrhodamine, chlororhodamine, methylrhodamine, sulforhodamine, and thiorhodamine, cyanine, indocarbocyanine, oxacarbocyanine, thiacarbocyanine, merocyanine, cyanine 2, cyanine 3, cyanine 3.5, cyanine 5, cyanine 5.5, cyanine 7, oxadiazole derivatives, pyridyloxazole, nitrobenzoxadiazole, benzoxadiazole, pyrene derivatives, cascade blue, oxazine derivatives, Nile red, Nile blue, cresyl violet, oxazine 170, acridine derivatives, proflavin, acridine orange, acridine yellow, arylmethine derivatives, auramine, crystal violet, malachite green, tetrapyrrole derivatives, porphin, phtalocyanine, and bilirubin. Exemplary quenchers include black hole quenchers, such as BHQ-0, BHQ-1, BHQ-2, BHQ-3; ATTO quenchers, such as ATTO 540Q, ATTO580Q, and ATTO612Q; and the like.

[00153] The fluorophores that may be used with the invention are not limited to any of the fluorophores described herein. For example, fluorophores with improved properties are continually developed, and these fluorophores could readily be used with the methods provided in this disclosure. Such improved fluorophores include quantum dots, which may emit energy at different wavelengths after being excited at a single wavelength. The advantage of using such fluorophores is that only a single excitation source is needed, but many different signals may be quantified, for example in terms of color and intensity. Moreover, fluorophores with narrow emission spectra would be particularly useful with the method described herein, as such fluorophores could be included in multiplex assays with minimal or no overlap between their

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emission spectra, thereby offering many more "colors" and boosting significantly the overall number of coded analytes.

[00154] In some cases, one or more reagents are lyophilized. Any suitable reagent may be lyophilized. For example, probes, primers, enzymes, antibodies, or any other reagent used for detection may be lyophilized. In some cases, a sample comprising an analyte may also be lyophilized. Lyophilization may be useful, for example, when distributing reagents and/or samples in developing regions where access to cold storage is expensive, not readily available, or not reliably available. In one example, lyophilized reagents comprise any probe or analyte-specific reagent described in this disclosure or otherwise suitable for use with the invention. In another example, lyophilized reagents comprise PCR primers. In yet another example, lyophilized reagents comprise reagents suitable for performing a PCR reaction.

V. Analytical Techniques and Instrumentation

[00155] The methods provided herein are suitable for use with a variety of detection methods. For example, the methods may be applied using an analytical technique that measures the wavelength and intensity of a fluorescent signal. This may be accomplished by measuring the intensity of a signal across a spectrum of wavelengths, or by using band pass filters that restrict the passage of certain wavelengths of light, thereby allowing only light of certain wavelengths to reach a photodetector. Many real-time PCR and quantitative PCR instruments comprise an excitation light source and band pass filters that enable the detection of fluorescent signals in four colors (e.g., blue, green, yellow, and red). Therefore, the methods of the invention can be readily applied using instruments widely used in the art. Importantly, the methods and compositions provided herein may be used to detect multiple analytes by obtaining a cumulative measurement on a single solution. No separation is necessary. The invention does not require the use of beads or a solid phase. Of course, one of ordinary skill in the art would understand that the invention could be used with separation, beads, or a solid phase, if desired.

[00156] **Fig. 5** shows two examples of instrumental configurations that may be used to obtain cumulative measurements useful for the methods of the invention. With reference to **Fig. 5A**, an instrument with a detector configured to detect both wavelength and intensity (e.g., a fluorometer) is shown**.** With reference to **Fig. 5A**, at least one excitation light source **502** is

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directed into a chamber **503** containing analytes and reagents that generate a signal in the presence of the analytes **504.** As described elsewhere, the analyte may be a nucleic acid and the reagents generating the signal may be hybridization probes comprising a fluorophore and a quencher. If the analyte is present, an emission signal **505** is generated. In the configuration shown in **Fig. 5A**, the wavelength and intensity of this emission signal are measured across a spectrum by a detector capable of generating a fluorescence emission spectrum **506.**

[00157] The wavelength and intensity may also be determined using a combination of a photodetector and band pass filters. This configuration is used in several thermal cyclers known in the art. With reference to **Fig. 5B**, an instrument with band pass filters and a photodetector is depicted**.** With reference to **Fig. 5B**, at least one excitation light source **508** is directed into a chamber **509** containing analytes and reagents that generate a signal in the presence of the analytes **510.** As described elsewhere, the analyte may be a nucleic acid and the reagents generating the signal may be hybridization probes comprising a fluorophore and a quencher. If the analyte is present, an emission signal **511** is generated. In the configuration shown in **Fig. 5B**, band pass filters **512, 513,** and **514** are used to restrict the passage of light to light within certain ranges of wavelengths. For example band pass filter **512** may restrict the passage of light to light within wavelength range 1. Band pass filter **513** may restrict the passage of light to light within wavelength range 2. Band pass filter **514** may restrict the passage of light to light within wavelength range 3. Any number of band pass filters (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more) may be used with the invention. In addition, the positions of the band pass filters in **Fig. 5B** are not meant to be limiting. In some cases, emitted light may be passed through more than one band pass filter at any time. In other cases, the band pass filters may be configured so that all emitted light passes through only one band pass filter at a time. After passage through a band pass filter, filtered light **515, 516,** and **517** is detected by a photodetector. For example, filtered light **515** may be light within wavelength range 1, filtered light **516** may be light within wavelength range 2, and filtered light **517** may be light within wavelength range 3. The intensity of each of the filtered lights **515, 516,** and **517** may then be quantified by a photodetector **518.**

[00158] The methods described in this disclosure are compatible with a variety of amplification methods, including quantitative PCR (qPCR) methods, end point PCR methods,

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reverse transcriptase PCR, and digital PCR methods. Digital PCR methods (e.g., DROPLET DIGITAL (BIORAD) and DYNAMIC ARRAY (FLUIDIGM)) produce highly sensitive quantification of polynucleotide copy numbers. The methods provided herein can be easily integrated into these systems to significantly expand their throughput by allowing multiplexing in a droplet or a dynamic array.

[00159] Similarly, the methods described in this disclosure may be applied in a real-time PCR assay. For example, real-time data may be fully recorded as the PCR runs to completion. The end-point values may then be decoded to indicate the presence or absence of analytes. The individual cycle thresholds (Ct) values may be analyzed for each color by detecting the maxima of the second derivative of the real-time curve in that color. As a particular analyte is amplified, fluorophores are released from its corresponding probes in the same ratios as the color multiplicities in the coding of that sequence. Hence, e.g. a code of 1100 will have the same Ct in blue and green, while a code of 1400 will have its green Ct precede its blue Ct by 2 cycles. This additional data enables determination of the identity and starting quantity of each analyte.

[00160] The methods described herein may also be used directly on a tissue sample. For example, a tissue sample may be obtained and photolithographic methods may be used to build wells directly onto the tissue sample. The tissue sample may be fixed prior to building the wells. The tissue sample may then be analyzed by dispensing appropriate reagents into the wells in the tissue sample. The encoding and decoding methods described in this disclosure may be used for the multiplexed detection of analytes within the wells etched into the tissue. Each well may correspond to a single cell or a few cells, providing excellent spatial resolution when analyzing different portions of a tissue for an analyte. This method may be used to detect analytes in different areas of the same tissue.

[00161] In some cases, instruments may be modified or constructed, for example, to provide additional excitation light sources, at multiple wavelength, and/or to provide additional band pass filters or a capability of determining a complete spectrum. Including additional excitation sources would allow for the excitation of a larger variety of fluorophores. Including additional band pass filters, or modifying or constructing an instrument capable of determining an entire spectrum allows detection of a wider variety of emissions from fluorophores. These techniques can be used to increase the number of fluorophores that can be used with the methods

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described in this disclosure and, accordingly, to increase the number of analytes that be simultaneously detected.

[00162] In some cases, the methods described in this disclosure utilize end-point PCR methods. However, in some cases a result may be obtained before the end-point. This may be advantageous when, for example, results are needed as soon as possible. For example, in one case calibration experiments are performed in which different starting concentrations of an analyte are analyzed to determine the cycle number at which the signal becomes saturated, as a function of the starting amount of the analyte. Using this data, a computer monitoring a PCR reaction in real-time can be programmed to search for saturation up to the maximal cycle number according to the limit of detection (LOD) of the particular system. If there is no amplification of an analyte (e.g., other than a positive control, if present), by the maximal cycle number, the result for that analyte is negative. If there is amplification of the analyte by the maximal cycle number, the result for that analyte is positive and the saturation intensity in each color may be used to decode the result using the coding scheme. In both cases, there would be no need to run the PCR reaction beyond the number of cycles set by the LOD for that instrument. Although PCR has been used to illustrate this quantitative method, one of ordinary skill in the art will readily recognize that similar principles could be applied in any catalytic system where the starting amount of an analyte limits the rate of reaction, and the rate of reaction can be measured and reported by an evolution in a signal intensity over time.

VI. Compositions and Kits

[00163] This disclosure also provides compositions and kits for use with the methods described herein. The compositions may comprise any component, reaction mixture and/or intermediate described herein, as well as any combination. For example, the disclosure provides detection reagents for use with the methods provided herein. Any suitable detection reagents may be provided, including hybridization probes labeled with a fluorophore and a quencher and primers, as described elsewhere in the specification.

[00164] In some cases, compositions comprise reagents for the detection of at least seven analytes using four fluorophores. In some cases, compositions comprise reagents for the detection of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26,

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27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using four fluorophores. In some cases, compositions comprise reagents for the detection of at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using five fluorophores. In some cases, compositions comprise reagents for the detection of at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using six fluorophores. In some cases, compositions comprise reagents for the detection of at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using seven fluorophores.

[00165] In some cases the compositions comprise primers. The compositions may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 pairs of primers.

[00166] The invention also provides kits for carrying out the methods of the invention. Accordingly, a variety of kits are provided in suitable packaging. The kits may be used for any one or more of the uses described herein, and, accordingly, may contain instructions for detecting the presence or absence of each analyte of a plurality of analytes. A kit may comprise a coding scheme, or a decoding matrix, to assist the user in converting a cumulative measurement to a result indicating the presence or absence of each of a plurality of analytes. A kit may be a diagnostic kit, for example, a diagnostic kit suitable for the detection of any analyte, including the analytes recited herein. A kit may contain any of the compositions provided in this disclosure, including those recited above.

[00167] **Fig. 6** shows schematics of exemplary kits of the invention. With reference to **Fig. 6**, a kit comprising a kit body **601** is shown that contains reagents for the detection of analytes (e.g., polynucleotide probes comprising a fluorophore and a quencher) **602** and primers for the amplification of a nucleic acid **603.** The kit may also contain any other reagents, such as reagents suitable for performing an amplification reaction **604.** In the embodiment depicted in **601**, the reagents for the detection of analytes and the primers are each contained in a single volume (e.g., tube or a bottle). However, these reagents may also be provided in separate

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volumes, as depicted the kit body shown in **605.** The kit body **605** contains seven separate volumes. Three volumes (**606, 607,** and **608**) contain reagents for the detection of analytes (or mixtures of such reagents) and three volumes (**609, 610,** and **611**) contain primers (or mixtures of primers). The kit may also contain reagents suitable for performing an amplification reaction **604.**

[00168] **Fig. 6** is provided for illustrative purposes only. Any number of reagents for the detection of analytes (e.g., probes) and any number of primers may be included in a single tube or bottle, as appropriate for the application. For example, in some embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more reagents for the detection of analytes may be included in a single bottle. In other cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more primers for amplification may be included in a single bottle or tube. In some cases, primers and probes may be provided in the same bottle or tube. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more probes may be provided per pair of primers. In other cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more primers may be provided per pair of probes. The kits may contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more bottles or tubes. Control analytes, and their associated detection reagents and primers, may also be included in the kit. These may be included in a separate bottle or tube or, for example, included within the bottle or tube providing reagents for an amplification reaction **604**. As described elsewhere in the specification, any reagent in the kits may be lyophilized.

VII. Systems and Software

[00169] The methods of this disclosure may also be performed as part of a system. For example, one or more steps of the methods described herein may be performed by software contained on a computer readable medium. The software may be used to encode analytes, check

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for degeneracy, reduce or eliminate degeneracy, and/or produce a decoding matrix. The software may also be used to convert cumulative measurements made on an instrument (e.g., a PCR machine) into information concerning the presence or absence of an analyte. For example, with reference to **Fig. 4**, a computer **401** may be connected to an input device **403**, a display **402**, and a printer **404**. The computer may execute one or more of the steps of any of the methods described in this disclosure, including, for example, encoding methods **410**, analysis methods **420**, and decoding methods **430.** A computer may reduce or eliminate degeneracy, for example as part of the encoding methods **410.** Similarly, a computer may generate a coding schme that is non-degenerate by design using, for example, mathematical iteration. A computer may also be connected to a network **405**. The network may be a local network and/or the Internet. The network may be used to perform the methods of the invention on multiple computers in different locations. For example, one computer may perform the encoding, another computer may perform the measuring, and yet another computer may perform the decoding. A computer may perform any or all of these methods. The network may be used to transmit information obtained using the methods described in this disclosure throughout the world, for example across a border.

[00170] In one example, the software is embedded in the instrument making the measurement, such as a thermal cycler. In another example, the software is installed on a computer attached to the instrument making the measurement, such as a computer attached to a thermal cycler. In another example, the software is in the "cloud" -- i.e., on a computer that another computer may communicate with through a network.

[00171] Each of the elements described above may be connected to a controller that communicates with each of the elements and coordinates their actions. For example, a controller may initiate the encoding of a series of analytes into values of color and intensity. As described throughout this disclosure, the controller can execute software to reduce or eliminate degeneracy by removing one or more potential analyte codes from the encoding matrix. The controller may then automatically order probes corresponding to this code from a manufacturer of probes. Alternatively, the controller may operate an oligonucleotide synthesis instrument that can synthesize the appropriate probes. After analysis of the sample on the instrument (which may also be automated), the controller may process the results of the analysis using the decoding matrix. The controller may then provide these results to a user.

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VIII. Services

[00172] The methods provided herein may also be performed as a service. For example, a service provider may obtain the identity of a plurality of analytes that a customer wishes to analyze. The service provider may then encode each analyte to be detected by any of the methods described herein and provide appropriate reagents to the customer for the assay. The customer may perform the assay and provide the results to the service provider for decoding. The service provider may then provide the decoded results to the customer. The customer may also encode analytes, generate probes, and/or decode results by interacting with software installed locally (at the customer's location) or remotely (e.g., on a server reachable through a network). Exemplary customers include clinical laboratories, physicians, manufacturers of food and consumer products, industrial manufacturers (e.g., petroleum companies) and the like. A customer or party may be any suitable customer or party with a need or desire to use the methods, systems, compositions, and kits of the invention.

EXAMPLES

Example 1: General Materials & Methods

1. General Reagents

[00173] TE Buffer, pH7 (LIFE TECHNOLOGIES, Carlsbad, CA); UltraPure RNAse-free Water (LIFE TECHNOLOGIES, Carlsbad, CA); Taq 5x Master Mix (FISHER SCIENTIFIC COMPANY, Tustin, CA).

2. DNA Sequences, Primers, and Probes

[00174] Five nucleic acids from organisms of clinical relevance were chosen for the exemplary study: (1) human immunodeficiency virus 1 (HIV-1); (2) *Plasmodium falciparum* (Malaria); (3) herpes simplex virus-2 (HSV-2); (4) *Mycobacterium tuberculosis* (TB); (5) and dengue virus type 3 (dengue fever).

[00175] Two regions of diagnostic relevance from the HIV-1 genome, p17 and poly-protease, were selected from the Los Alamos National Laboratory HIV-1 reference sequence. A diagnostic sequence from the *Plasmodium falciparum* ChR7 gene was obtained from the UCSC *Plasmodium falciparum* Genome Browser. A sequence for Herpes Simplex Virus-2 was synthesized from the sequence obtained from the European Molecular Biology Library.

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Similarly a diagnostic sequence for the rpoB gene in *Mycobacterium tuberculosis* was synthesized from a sequence obtained from the European Molecular Biology Library. A PCR diagnostic sequence for Dengue Virus Type 3 was obtained from the National Institute of Health genetic sequence database.

[00176] Oligonucleotides were synthesized by INTEGRATED DNA TECHNOLOGIES (IDT). Probes and primer pairs were chosen for each analyte using OLIGOANALYZER, from IDT. Sense probes containing a fluorophore at the 5' end and a quencher at the 3' end were synthesized for all analytes. All oligonucleotides were lyophilized and reconstituted in TE buffer before use. Tables 9 - 14 show sequence information, target sequence, primers, and probes for each of the six analytes.

Table 9. HIV-1 polyprotease sequence information.

Sequence Information	HIV-1 Poly protease 198mer synthesized from bases 2253 – 2550
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (3' to 5')	GGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGA GTTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTT ATCAAAGTAAGACAGTATGATCAGATACTCATAGAAATCTGTGGACATAA AGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGG
Forward Primer (3' to 5')	GGAAGCTCTATTAGATACAGGAGCAG
Reverse Primer (3' to 5')	CCAATTATGTTGACAGGTGTAGGTCC
Probe 1 (3' to 5')	**/56-FAM/**TGAGTTTGCCAGGAAGATGGAAACCA/3BHQ_1/

Table 10. HIV p17 sequence information.

Sequence Name	HIV-1 P17 199mer synthesized from bases 790 – 1186
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target	CAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATA

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sequence (3' to 5')	TAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAG ACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAG AAAAAAGCACAGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGTCA
Forward Primer (3' to 5')	CAGCTACAACCATCCCTTCAGACA
Reverse Primer (3' to 5')	TGACCTGATTGCTGTGTCCTGTGT
Probe 1 (3' to 5')	**/56-FAM/**AGCAACCCTCTATTGTGTGCATCAAAGG/3BHQ_1
Probe 2 (3' to 5')	**/5Cy3/**AAAGCACAGCAAGCAGCAGCTGA/3BHQ_2/

Table 11. *Plasmodium* ChR7 sequence information.

Sequence Name	*Plasmodium* ChR7 199mer synthesized from bases 1139138 - 1141223
Source	UCSC Plasmodium falciparum Genome Browser Gateway
Target sequence (3' to 5')	GCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAG AGGGAGCCTGAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTA AATTACCCAATTCTAAAGAAGAGAGGTAGTGACAAGAAATAACAATGCAA GGCCAATTTAAAACCTTCCCAGAGTAACAATTGGAGGGCAAGTCTGGTG
Forward Primer (3' to 5')	GCCTAACATGGCTATGACGGGTAA
Reverse Primer (3' to 5')	CACCAGACTTGCCCTCCAATTGTT
Probe 1 (3' to 5')	**/56-FAM/**ATTCCGGAGAGGGAGCCTGAGAAATA/3BHQ_1/
Probe 2 (3' to 5')	**/56-ROXN/**AAGGAAGGCAGCAGGCGCGTAAATTA/3BHQ_2/

Table 12. Herpes simplex virus 2 sequence information.

Sequence Name	HSV-2 193mer synthesized from HSV-2 genome
Source	EMBL Bank AJ303204

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Target sequence (3' to 5')	TCAGCCCATCCTCCTTCGGCAGTATGGAGGGTGTCGCGGCGGCGAGCCGC CGTCCCCAAAGACGTGCGGGTCGTACACGTACACGTACCAGGGCGGCGGG CCTCCGACCCGGTACGCTCTCGTAAATGCTTCCCTGCTGGTGCCGATCTG GGACCGCGCCGCGGAGACATTCGAGTACCAGATCGAACTCGG
Forward Primer (3' to 5')	TCAGCCCATCCTCCTTCGGCAGTAT
Reverse Primer (3' to 5')	CCGAGTTCGATCTGGTACTCGAATGT
Probe 1 (3' to 5')	**/56-FAM/**AAAGACGTGCGGGTCGTACACGTACA/3BHQ_1/
Probe 2 (3' to 5')	**/5Cy5/**TAAATGCTTCCCTGCTGGTGCCGAT/3IAbRQSp/

Table 13. *Mycobacterium tuberculosis* rpoB sequence information

Sequence Name	Mycobacterium Tuberculosis rpoB 200mer synthesized from Mycobacterium tuberculosis rpoB genome
Source	EMBL Bank GQ395623
Target sequence (3' to 5')	GAGTGCAAAGACAAGGACATGACGTACGCGGCCCCGCTGTTCGTCACGGC CGAGTTCATCAACAACAACACCGGCGAGATCAAGAGCCAGACGGTGTTCA TGGGTGACTTCCCGATGATGACCGAGAAGGGCACCTTCATCATCAACGGC ACCGAGCGCGTCGTGGTCAGCCAGCTGGTCCGCTCGCCCGGTGTGTACTT
Forward Primer (3' to 5')	GAGTGCAAAGACAAGGACATGACG
Reverse Primer (3' to 5')	AAGTACACACCGGGCGAGC
Probe 1 (3' to 5')	**/56-FAM/**CGCTGTTCGTCACGGCCGAGTTCAT/3BHQ_1/
Probe 2 (3' to 5')	**/5Cy3/**AGATCAAGAGCCAGACGGTGTTCATG/3BHQ_2/
Probe 3 (3' to 5')	**/5Cy5/**AAGGGCACCTTCATCATCAACGGCA/3IAbRQSp/

Table 14. Dengue virus type 3 sequence information.

Sequence Name	Dengue Virus Type 3 200mer synthesized from Dengue Virus Type 3 genome
Source	GenBank M93130
Target sequence	ATGCCAACTGTGATTGAGCACTTAGAAAGACTACAAAGGAAACATGGAGG

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(3' to 5')	AATGCTTGTGAGAAATCCACTCTCACGAAACTCCACGCACGAAATGTATT GGATATCCAATGGTACAGGCAACATCGTCTCATTCACAATGACACACAGG AGACCCACCATAGAGAAAGATGTGGATTTAGGAGCAGGAACCCGACATGT
Forward Primer (3' to 5')	ATGCCAACTGTGATTGAGCACT
Reverse Primer (3' to 5')	ACATGTCGGGTTCCTGCTCCTAAA
Probe 1 (3' to 5')	**/56-FAM/**ACAAAGGAAACATGGAGGAATGCTTGTGA/3BHQ_1/
Probe 2 (3' to 5')	**/5Cy3/**ACTCTCACGAAACTCCACGCACGAAA/3BHQ_2/
Probe 3 (3' to 5')	**/56-ROXN/**ACAATGACACACAGGAGACCCACCAT/3BHQ_2/
Probe 4 (3' to 5')	**/5Cy5/**GGATATCCAATGGTACAGGCAACATCGT/3IAbRQSp/

3. Polymerase Chain Reactions

[00177] All PCR reactions were performed in a ROCHE 480 LIGHTCYCLER instrument. Forty-five thermal cycles were performed, with a 60 second hot-start at 95°C. The cycling conditions for denaturation, annealing, and extension were 45 seconds, 50 seconds, and 60 seconds at 95°C, 65°C, and 70°C, respectively. Each experiment was run in quintuplicate, with a reaction volume of 15 μL. Fluorescence measurements were obtained in the following channels after every annealing step: 483 nm to 533 nm (FAM), 523 nm to 568 nm (Cy3), 558 nm to 610 nm (ROX), and 615 nm to 670 nm (Cy5). Fluorescence measurements were also obtained after the hot-start and the end of the 45 thermal cycles. The change in the fluorescence intensity between these two measurements (after hot-start and at the end of the 45 cycles) determined the end-point fluorescent signal.

Example 2: Simultaneous Detection of Polynucleotide Markers from Pathogens Based on an Encoding Method Using More Than One Color Per Analyte

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[00178] This example describes the simultaneous detection of up to four polynucleotides selected from HIV (2 polynucleotides), HSV-2, *Mycobacterium*, *Plasmodium*, and dengue virus. Fourteen PCR reactions were assembled, using the reagents described in Table 15.

Table 15. Reagents for 14 PCR reactions.

Reaction No.	Type of Reagent	Reagent	Concentration	Volume
1		UltraPure Water	-	34 µL
		Taq 5x Master Mix	-	42 µL
	Template	Poly Protease Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 nM	2 µL
		Protease RVS Primer	1 nM	2 µL
	Probe	Poly Protease Probe 1	1 nM	2 µL
2		UltraPure Water	-	28 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 nM	2 µL
		Protease RVS Primer	1 nM	2 µL
	Probe	Poly Protease Probe 1	1 nM	2 µL
3		UltraPure Water	-	32 µL
		Taq 5x Master Mix	-	42 µL
	Template	Malaria Template	15 nM	2 µL
	Primers	Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
	Probes	Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
4		UltraPure Water	-	26 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
	Probes	Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
5		UltraPure Water	-	32 µL
		Taq 5x Master Mix	-	42 µL

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	Template	Herpes Template	17 nM	2 µL
	Primers	Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
	Probes	Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
6		UltraPure Water	-	26 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
	Probes	Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
7		UltraPure Water	-	30 µL
		Taq 5x Master Mix	-	42 µL
	Template	TB Template	16 nM	2 µL
	Primers	TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
8		UltraPure Water	-	30 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
9		UltraPure Water	-	28 µL
		Taq 5x Master Mix	-	42 µL
	Template	Dengue Virus Template	17 nM	2 µL
	Primers	Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL

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10		UltraPure Water	-	22 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
11		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease RVS Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
12		UltraPure Water	-	2 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL

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		Malaria RVS Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
13		UltraPure Water	-	12 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
14		UltraPure Water	-	10 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
		Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL

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		Malaria Probe 2	1 μM	2 μL
		Dengue Virus Probe 1	1 μM	2 μL
		Dengue Virus Probe 2	1 μM	2 μL
		Dengue Virus Probe 3	1 μM	2 μL
		Dengue Virus Probe 4	1 μM	2 μL

[00179] Referring to Table 15, reactions 1, 3, 5, 7, and 9 (containing HIV polyprotease, *Plasmodium*, HSV, *Mycobacterium*, and dengue analytes, respectively) were positive controls that provided baseline fluorescence intensity for each polynucleotide analytes alone, in the absence of other analytes. The change in fluorescence intensity in each color was used to provide the expected cumulative intensity level for each color in the chromatogram **(Fig. 3)**, as described more fully below. Reactions 2, 4, 6, 8, and 10 were negative controls used to determine the extent of non-specific amplification. In these reactions, the primers provided were not specific for the analytes provided.

[00180] Experiments 11, 12, 13, and 14 were multiplex detection experiments. Encoding was performed as indicated in Table 16. The measured fluorescence intensity for each assay, in each color, was plotted as black circles in the chromatogram of **Fig. 3.**

Table 16. Coding scheme for detection of pathogens.

	FAM	**Cy3**	**ROX**	**Cy5**
HIV poly-protease	1	0	0	0
HIV p17	1	1	0	0
Plasmodium	1	0	1	0
HSV	1	0	0	1
Mycobacterium	1	1	0	1
Dengue Virus	1	1	1	1

[00181] To construct the chromatogram, the signals of the positive controls (reactions 1, 3, 5, 7, and 9) were used to assemble every possible combination of present sequences, in each color. The cumulative signals were plotted in a diagram, called a "chromatogram" (**Fig. 3**). Cumulative signals corresponding to all possible combinations of the same rank in the same color were organized into their own "band." Doing this for all four colors produced a level and band structure, against which the experimental results of each combination (reactions 11, 12, 13,

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and 14) could be evaluated. **Fig. 3** shows three replicates of a chromatogram constructed using the positive control samples, with experimental results 4112, 3121, and 3102 superimposed on the chromatogram. The experimental results are indicated by black dots.

[00182] The intensity value for each color in reactions 11-14 was measure in arbitrary fluorescence units (AFU), plotted on the chromatogram, and then assigned to a band based on two criteria. First, if an experimental result was within a band, then the result was assigned to the multiplicity of the band (e.g., the Cy3 value of 1 in the left-most chromatogram of **Fig. 3**). Second, if an experimental result was between two bands, a two-step analysis was employed. If the experimental result was between two legitimate results, the experimental result was assigned to the closest band. (e.g., the Cy5 value of 2 in the right-most chromatogram of **Fig. 3**). If the experimental result was between a legitimate result and an illegitimate result, the result was assigned to the band with the legitimate result (e.g., the FAM value of 3 in the middle chromatogram of **Fig. 3**). **Fig. 8** schematically shows a legitimate result of 4112 (top) indicating the presence of HIV polyprotease, *Plasmodium*, herpes simplex, and *Mycobacterium*; and an illegitimate result of 4110 (bottom), which cannot be decoded and indicates a malfunction in the assay.

[00183] Using these criteria, the output of Experiment 12 was designated 4112, which was the correct answer. The output of Experiment 14 could have been designated as 2121 or 3121, but 2121 is illegitimate, so the result must be 3121, which is the correct answer. The output of Experiment 13 could be designated as 3102 or 3101, both of which are legitimate, but the result is clearly closer to 2 than 1 in the Cy5 channel, so 3102, the correct answer, was designated as the result. These results provide experimental validation of the methods of the provided herein.

[00184] The results indicate a trend of slightly lower combined signal than what would be expected from a simple summation of positive-control signals. This may be because the combination of signals is not perfectly linear. This may be a result of additional effects for which the chromatogram construction does not account for at present. These effects may be related to the absolute concentration of quenchers. The higher the absolute concentration of quenchers, the larger the percentage of reaction volume they quench, so that the same percentage of released fluorophores fails to contribute as much as expected to the cumulative signal. One

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solution to this problem is to use low concentrations of probes, so that the percentile loss in signal is negligible, regardless of how much quencher is released.

[00185] **Fig. 3** shows that each rank containing multiple combinations has a relatively wide band. The width of the band is ultimately the difference between the highest and lowest cumulative signals for the positive control samples. If all positive controls in the particular color produced exactly the same fluorescence signal, then the width of each band would be zero. Since instead those signals are somewhat different, the resulting bands are relatively wide. However, there is a simple means to tighten the bands. Instead of loading all probes at the same concentration, as was done for the experiment above, the concentration of each probe can be adjusted so that each of the resulting positive-control signals is the same as the others in the same color. This approach would significantly tighten the bands, making it easier to determine the multiplicity of a signal for a sample containing analytes.

Example 3: Simultaneous Detection of Polynucleotide Markers from Pathogens Based on an Encoding Method Using One Color Per Analyte and Different Intensities

[00186] This example describes the simultaneous detection of up to three polynucleotides selected from dengue virus, HIV p17, and HIV polyprotease. The coding scheme was as depicted in Table 11. As shown in Table 17, each analyte was encoded as a single value of fluorescence intensity in a single color. In this case, as described elsewhere in this disclosure, the coding scheme is non-degenerate by design. This is achieved by assigning an intensity value to each analyte that is equal to the cumulative intensity values for the prior analytes, plus one. Of course, as described elsewhere in the specification, the initial assigned value can be greater than one, for example to better distinguish over noise. Similarly, the values assigned to each analyte can be greater than the cumulative intensity values for the prior analytes plus one, for example, to increase the separation between the intensities and enable more simple assignment of intensities.

Table 17. Coding scheme for detection of dengue virus, HIV polyprotease, and HIV p17.

	FAM
Dengue Virus	1
HIV p17	2

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HIV polyprotease	4

[00187] The same reagents (e.g., templates, primers, and probes) used in Example 2 were used here. The fluorescence signals generated by the respective probes in positive-control end-point PCR reactions were measured and used to calculate the probe concentrations that would produce 1x, 2x, and 4x signal intensities. Experiments were then set up to detect the seven non-null combinations of analyte occurrences. Tables 18-24 provide the PCR reaction components for each of these mixtures.

Table 18. Binary FAM Experiment 1 Cocktail: HIV Polyprotease, HIV p17, and dengue virus

Reagents	Concentration	Volume Added
UltraPure Water	-	50µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	4µL
P17 Template	15nM	4µL
Dengue Virus Template	17nM	4µL
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 19. Binary FAM Experiment 2 Cocktail: dengue virus and HIV p17

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Reagents	Concentration	Volume Added
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	-
P17 Template	15nM	4µL
Dengue Virus Template	17nM	4µL
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 20. Binary FAM Experiment 3 Cocktail: dengue virus and HIV polyprotease

Reagents	Concentration	Volume Added
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	4µL
P17 Template	15nM	-

Dengue Virus Template	17nM	4µL
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 21. Binary FAM Experiment 4 Cocktail: HIV polyprotease and HIV p17

Reagents	Concentration	Volume Added
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	4µL
P17 Template	15nM	4µL
Dengue Virus Template	17nM	-
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	Concentration	Volume Added

Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 22. Binary FAM Experiment 5 Cocktail: dengue virus

Reagents	Concentration	Volume Added
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	-
P17 Template	15nM	-
Dengue Virus Template	17nM	4µL
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 23. Binary FAM Experiment 6 Cocktail: HIV p17

Reagents	Concentration	Volume Added
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added

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Poly Protease Template	17nM	-
P17 Template	15nM	4μL
Dengue Virus Template	17nM	-
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1μM	4μL
Poly Protease RVS Primer	1μM	4μL
P17 FWD Primer	1μM	4μL
P17 RVS Primer	1μM	4μL
Dengue Virus FWD Primer	1μM	4μL
Dengue Virus RVS Primer	1μM	4μL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4μL
P17 Probe 1	400nM	4μL
Dengue Virus Probe 1	200nM	4μL

Table 24. Binary FAM Experiment 7 Cocktail: HIV polyprotease

Reagents	Concentration	Volume Added
UltraPure Water	-	58μL
Taq 5x Master Mix	-	25μL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	4μL
P17 Template	15nM	-
Dengue Virus Template	17nM	-
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1μM	4μL
Poly Protease RVS Primer	1μM	4μL
P17 FWD Primer	1μM	4μL
P17 RVS Primer	1μM	4μL
Dengue Virus FWD Primer	1μM	4μL

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Dengue Virus RVS Primer	1μM	4μL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4μL
P17 Probe 1	400nM	4μL
Dengue Virus Probe 1	200nM	4μL

[00188] The total fluorescence signal of each experiment was plotted on a chromatogram (**Fig. 7**) that was constructed as described above. The width of the bands around each positive control measurement is the propagated uncertainty of the 1X measurement. That uncertainty was equated to the standard deviation of the fluorescence signals of the last five PCR cycles in saturation.

[00189] **Fig. 7** shows the results of this experiment. The three right-most results show the measured intensities for dengue virus alone (DEN), HIV p17 alone (P17), and HIV polyprotease (TPP). The assigned intensities for each of these analytes were as indicated in Table 11, and **Fig. 7** confirms that the three analytes yielded the expected intensity results when alone (see DEN, P17, and TPP). At the end of the assay, essentially all of the probes have been hydrolyzed, releasing essentially all of the fluorophores, which emit a signal. A fixed amount of a fluorophore produces a fixed and predictable amount of signal. Therefore a target that is present should generally produce a fixed amount of signal, as determined by the amount of probe added to the reaction volume.

[00190] As expected, the intensity values for the combinations of analytes are equivalent to the sum of the assigned intensities. Since this coding scheme is designed to be non-degenerate, each result can be unambiguously decoded into the presence or absence of a particular analyte. For example, starting from the left side of **Fig. 7**, a result of 7 indicates the presence of all three analytes. A result of 3 indicates the presence of dengue virus and HIV p17 analytes, and the absence of HIV polyprotease. A result of 5 indicates the presence of dengue virus and HIV polyprotease, and the absence of HIV p17. Finally a result of 6 indicates the presence of HIV p17 and HIV polyprotease, and the absence of dengue virus. This coding scheme can be extended to encode additional analytes, as described elsewhere in this disclosure.

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Supplemental Tables and Figures

Table S1 HIV-1 Poly Protease Sequence Information

Sequence Information	HIV-1 Poly protease 198mer synthesized from bases 2253 – 2550
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (5' to 3')	GGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGA GTTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTT ATCAAAGTAAGACAGTATGATCAGATACTCATAGAAATCTGTGGACATAA AGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGG
Forward Primer (5' to 3')	GGAAGCTCTATTAGATACAGGAGCAG
Reverse Primer (5' to 3')	CCAATTATGTTGACAGGTGTAGGTCC
Probe 1 (5' to 3')	/**56-FAM**/TGAGTTTGCCAGGAAGATGGAAACCA/3BHQ_1/

Table S2 HIV-1 P17 Sequence Information

Sequence Name	HIV-1 P17 199mer synthesized from bases 790 – 1186
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (5' to 3')	CAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATA TAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAG ACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAG AAAAAAGCACAGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGTCA
Forward Primer (5' to 3')	CAGCTACAACCATCCCTTCAGACA
Reverse Primer (5' to 3')	TGACCTGATTGCTGTGTCCTGTGT
Probe 1 (5' to 3')	/**56-FAM**/AGCAACCCTCTATTGTGTGCATCAAAGG/3BHQ_1
Probe 2 (5' to 3')	/**5Cy3**/AAAGCACAGCAAGCAGCAGCTGA/3BHQ_2/

Table S3 Malaria ChR7 Sequence Information

Sequence Name	Malaria ChR7 199mer synthesized from bases 1139138 - 1141223
Source	UCSC Plasmodium falciparum Genome Browser Gateway
Target sequence (5' to 3')	GCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAG AGGGAGCCTGAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTA AATTACCCAATTCTAAAGAAGAGAGGTAGTGACAAGAAATAACAATGCAA GGCCAATTTAAAACCTTCCCAGAGTAACAATTGGAGGGCAAGTCTGGTG
Forward Primer (5' to 3')	GCCTAACATGGCTATGACGGGTAA
Reverse Primer (5' to 3')	CACCAGACTTGCCCTCCAATTGTT
Probe 1 (5' to 3')	/**56-FAM**/ATTCCGGAGAGGGAGCCTGAGAAATA/3BHQ_1/

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Probe 2 (5' to 3')	/**56-ROXN**/AAGGAAGGCAGCAGGCGCGTAAATTA/3BHQ_2/

Table S4 Herpes Simplex Virus-2 Sequence Information

Sequence Name	HSV-2 193mer synthesized from HSV-2 genome
Source	EMBL Bank AJ303204
Target sequence (5' to 3')	TCAGCCCATCCTCCTTCGGCAGTATGGAGGGTGTCGCGGCGGCGAGCCGC CGTCCCCAAAGACGTGCGGGTCGTACACGTACACGTACCAGGGCGGCGGG CCTCCGACCCGGTACGCTCTCGTAAATGCTTCCCTGCTGGTGCCGATCTG GGACCGCGCCGCGGAGACATTCGAGTACCAGATCGAACTCGG
Forward Primer (5' to 3')	TCAGCCCATCCTCCTTCGGCAGTAT
Reverse Primer (5' to 3')	CCGAGTTCGATCTGGTACTCGAATGT
Probe 1 (5' to 3')	/**56-FAM**/AAAGACGTGCGGGTCGTACACGTACA/3BHQ_1/
Probe 2 (5' to 3')	/**5Cy5**/TAAATGCTTCCCTGCTGGTGCCGAT/3IAbRQSp/

Table S5 Tuberculosis rpoB Sequence Information

Sequence Name	Mycobacterium Tuberculosis rpoB 200mer synthesized from Mycobacterium tuberculosis rpoB genome
Source	EMBL Bank GQ395623
Target sequence (5' to 3')	GAGTGCAAAGACAAGGACATGACGTACGCGGCCCCGCTGTTCGTCACGGC CGAGTTCATCAACAACAACACCGGCGAGATCAAGAGCCAGACGGTGTTCA TGGGTGACTTCCCGATGATGACCGAGAAGGGCACCTTCATCATCAACGGC ACCGAGCGCGTCGTGGTCAGCCAGCTGGTCCGCTCGCCCGGTGTGTACTT
Forward Primer (5' to 3')	GAGTGCAAAGACAAGGACATGACG
Reverse Primer (5' to 3')	AAGTACACACCGGGCGAGC
Probe 1 (5' to 3')	/**56-FAM**/CGCTGTTCGTCACGGCCGAGTTCAT/3BHQ_1/
Probe 2 (5' to 3')	/**5Cy3**/AGATCAAGAGCCAGACGGTGTTCATG/3BHQ_2/
Probe 3 (5' to 3')	/**5Cy5**/AAGGGCACCTTCATCATCAACGGCA/3IAbRQSp/

Table S6 Dengue Virus Type 3 Sequence Information

Sequence Name	Dengue Virus Type 3 200mer synthesized from Dengue Virus Type 3 genome
Source	GenBank M93130
Target sequence (5' to 3')	ATGCCAACTGTGATTGAGCACTTAGAAAGACTACAAAGGAAACATGGAGG AATGCTTGTGAGAAATCCACTCTCACGAAACTCCACGCACGAAATGTATT

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	GGATATCCAATGGTACAGGCAACATCGTCTCATTCACAATGACACACAGG AGACCCACCATAGAGAAAGATGTGGATTTAGGAGCAGGAACCCGACATGT
Forward Primer (5' to 3')	ATGCCAACTGTGATTGAGCACT
Reverse Primer (5' to 3')	ACATGTCGGGTTCCTGCTCCTAAA
Probe 1 (5' to 3')	/**56-FAM**/ACAAAGGAAACATGGAGGAATGCTTGTGA/3BHQ_1/
Probe 2 (5' to 3')	/**5Cy3**/ACTCTCACGAAACTCCACGCACGAAA/3BHQ_2/
Probe 3 (5' to 3')	/**56-ROXN**/ACAATGACACACAGGAGACCCACCAT/3BHQ_2/
Probe 4 (5' to 3')	/**5Cy5**/GGATATCCAATGGTACAGGCAACATCGT/3IAbRQSp/

Table S7 Experiment 1 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	34µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	2µL
Poly Protease RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1µM	2µL

Table S8 Experiment 2 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	28µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Malaria Template	15nM	2µL
Herpes Template	17nM	2µL
TB Template	16nM	2µL
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	2µL

Poly Protease RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1µM	2µL

Table S9 Experiment 3 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	32µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Malaria Template	15nM	2µL
Primers	**Concentration**	**Volume Added**
Malaria FWD Primer	1µM	2µL
Malaria RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Malaria Probe 1	1µM	2µL
Malaria Probe 2	1µM	2µL

Table S10 Experiment 4 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	26µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Herpes Template	17nM	2µL
TB Template	16nM	2µL
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Malaria FWD Primer	1µM	2µL
Malaria RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Malaria Probe 1	1µM	2µL

Malaria Probe 2	1µM	2µL

Table S11 Experiment 5 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	32µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Herpes Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Herpes FWD Primer	1µM	2µL
Herpes RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Herpes Probe 1	1µM	2µL
Herpes Probe 2	1µM	2µL

Table S12 Experiment 6 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	26µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Malaria Template	15nM	2µL
TB Template	16nM	2µL
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Herpes FWD Primer	1µM	2µL
Herpes RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Herpes Probe 1	1µM	2µL
Herpes Probe 2	1µM	2µL

Table S13 Experiment 7 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	30µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
TB Template	16nM	2µL
Primers	**Concentration**	**Volume Added**
TB FWD Primer	1µM	2µL
TB RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
TB Probe 1	1µM	2µL
TB Probe 2	1µM	2µL
TB Probe 3	1µM	2µL

Table S14 Experiment 8 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	30µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Malaria Template	15nM	2µL
Herpes Template	17nM	2µL
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
TB FWD Primer	1µM	2µL
TB RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
TB Probe 1	1µM	2µL
TB Probe 2	1µM	2µL
TB Probe 3	1µM	2µL

Table S15 Experiment 9 Cocktail

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Reagents	Concentration	Volume Added
UltraPure Water	-	28µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Dengue Virus FWD Primer	1µM	2µL
Dengue Virus RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Dengue Virus Probe 1	1µM	2µL
Dengue Virus Probe 2	1µM	2µL
Dengue Virus Probe 3	1µM	2µL
Dengue Virus Probe 4	1µM	2µL

Table S16 Experiment 10 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	22µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Malaria Template	15nM	2µL
Herpes Template	17nM	2µL
TB Template	16nM	2µL
Primers	**Concentration**	**Volume Added**
Dengue Virus FWD Primer	1µM	2µL
Dengue Virus RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Dengue Virus Probe 1	1µM	2µL
Dengue Virus Probe 2	1µM	2µL
Dengue Virus Probe 3	1µM	2µL
Dengue Virus Probe 4	1µM	2µL

Table S17 Experiment 11 Cocktail

Reagents	Concentration	Volume Added
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Malaria Template	15nM	2µL
Herpes Template	17nM	2µL
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	2µL
Poly Protease RVS Primer	1µM	2µL
Malaria FWD Primer	1µM	2µL
Malaria RVS Primer	1µM	2µL
Herpes FWD Primer	1µM	2µL
Herpes RVS Primer	1µM	2µL
Dengue Virus FWD Primer	1µM	2µL
Dengue Virus RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1µM	2µL
Malaria Probe 1	1µM	2µL
Malaria Probe 2	1µM	2µL
Herpes Probe 1	1µM	2µL
Herpes Probe 2	1µM	2µL
Dengue Virus Probe 1	1µM	2µL
Dengue Virus Probe 2	1µM	2µL
Dengue Virus Probe 3	1µM	2µL
Dengue Virus Probe 4	1µM	2µL

Table S18 Experiment 12 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	2µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**

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Poly Protease Template	17nM	2µL
Malaria Template	15nM	2µL
Herpes Template	17nM	2µL
TB Template	16nM	2µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	2µL
Poly Protease RVS Primer	1µM	2µL
Malaria FWD Primer	1µM	2µL
Malaria RVS Primer	1µM	2µL
Herpes FWD Primer	1µM	2µL
Herpes RVS Primer	1µM	2µL
TB FWD Primer	1µM	2µL
TB RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1µM	2µL
Malaria Probe 1	1µM	2µL
Malaria Probe 2	1µM	2µL
Herpes Probe 1	1µM	2µL
Herpes Probe 2	1µM	2µL
TB Probe 1	1µM	2µL
TB Probe 2	1µM	2µL
TB Probe 3	1µM	2µL

Table S19 Experiment 13 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	12µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Herpes Template	17nM	2µL
TB Template	16nM	2µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	2µL

Poly Protease RVS Primer	1µM	2µL
Herpes FWD Primer	1µM	2µL
Herpes RVS Primer	1µM	2µL
TB FWD Primer	1µM	2µL
TB RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1µM	2µL
Herpes Probe 1	1µM	2µL
Herpes Probe 2	1µM	2µL
TB Probe 1	1µM	2µL
TB Probe 2	1µM	2µL
TB Probe 3	1µM	2µL

Table S20 Experiment 14 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	10µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Malaria Template	15nM	2µL
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	2µL
Poly Protease RVS Primer	1µM	2µL
Malaria FWD Primer	1µM	2µL
Malaria RVS Primer	1µM	2µL
Dengue Virus FWD Primer	1µM	2µL
Dengue Virus RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1µM	2µL
Malaria Probe 1	1µM	2µL
Malaria Probe 2	1µM	2µL
Dengue Virus Probe 1	1µM	2µL

Dengue Virus Probe 2	1µM	2µL
Dengue Virus Probe 3	1µM	2µL
Dengue Virus Probe 4	1µM	2µL

Table S21 Binary FAM Experiment 1 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	50µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	4µL
P17 Template	15nM	4µL
Dengue Virus Template	17nM	4µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table S22 Binary FAM Experiment 2 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	-
P17 Template	15nM	4µL
Dengue Virus Template	17nM	4µL

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Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table S23 Binary FAM Experiment 3 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	4µL
P17 Template	15nM	-
Dengue Virus Template	17nM	4µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table S24 Binary FAM Experiment 4 Cocktail

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Reagents	Concentration	Volume Added
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	4µL
P17 Template	15nM	4µL
Dengue Virus Template	17nM	-
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table S25 Binary FAM Experiment 5 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	-
P17 Template	15nM	-
Dengue Virus Template	17nM	4µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL

Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table S26 Binary FAM Experiment 6 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	-
P17 Template	15nM	4µL
Dengue Virus Template	17nM	-
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table S27 Binary FAM Experiment 7 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**

Poly Protease Template	17nM	4µL
P17 Template	15nM	-
Dengue Virus Template	17nM	-
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

PATENT
Docket No.: 88DG-373535-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	)	
	)	Confirmation No.: 3145
Emil P. KARTALOV *et al.*	)	
	)	Examiner: Russell S. NEGIN
Application No.: 18/352,112	)	
	)	
Filed: July 13, 2023	)	Art Unit: 1672
	)	
For: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	)	***VIA Patent Center***
	)	

MAIL STOP ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AFTER ALLOWANCE UNDER 37 C.F.R. § 1.312

Dear Commissioner,

In response to the Notice to File Corrected Application Papers mailed October 30, 2024, the period for response ending on December 30, 2024, Applicant respectfully requests entry of this amendment under 37 C.F.R. 1.312 for the above-captioned patent application prior to issuance of the patent. A Replacement Drawing is also submitted under 37 C.F.R. 1.312 in connection with this Response.

Amendment to the Specification begin on page **2** of this paper.

Remarks begin on page **2** of this paper.

AMENDMENTS TO THE SPECIFICATION

Applicant submits herewith a Substitute Specification (marked and clean) in compliance with 37 C.F.R 1.121. The specification is amended at the following paragraphs to clarify **Fig. 5**:

Paragraph **[0051]**: has been amended to replace "Fig. 5 shows a schematic" with "Figs. 5A and 5B show schematics…"

Paragraphs **[00156]-[00157]** have been amended to rename **Fig. 5**, which shows the two examples of instrumental configurations to **Fig. 5A**, which references **501**, and **Fig. 5B**, which references **507**. Reference names **501** and **507** have been deleted in the Specification to reflect the Replacement Drawing showing the new **Figs. 5A and 5B** do not make reference to **501** and **507**.

No new matter has been introduced by this amendment.

REMARKS

Applicant respectfully requests entry of the clarifying amendments. The amendments do not change the scope of the claims and no new matter has been added.

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CONCLUSION

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. Prompt and favorable consideration of this Response is respectfully requested. A favorable response is awaited. It is believed that no fees are due in connection with this Rule 312 amendment. The Commissioner is authorized to charge any underpayment or credit any overpayment to Deposit account No. 50-4561 (Attorney Docket No. 88DG-373535-US)

Respectfully submitted,

SHEPPARD MULLIN RICHTER & HAMPTON LLP

By____/ David E. Heisey /_____
David E. Heisey
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Dated: November 12, 2024

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Customer No. 30764

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Placeholder Sheet for Supplemental Content

Application Number: 18352112 Document Date: 11/12/2024

The presence of this form indicates that the following document type was received in electronic format on the date identified above. This document is stored separately from the typical case contents which are stored in image format.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/352,112	07/13/2023	Emil P. KARTALOV	88DG-373535-US	3145

30764 7590 10/30/2024

Sheppard, Mullin, Richter & Hampton LLP
650 Town Center Drive, 10th Floor
Costa Mesa, CA 92626

EXAMINER
NEGIN, RUSSELL SCOTT

ART UNIT	PAPER NUMBER
1686	

NOTIFICATION DATE	DELIVERY MODE
10/30/2024	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):

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Application No. : 18352112
Applicant : Kartalov
Filing Date : 07/13/2023
Date Mailed : 10/30/2024

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Notice of Allowance Mailed

This application has been accorded an Allowance Date and is being prepared for issuance. The application, however, is incomplete for the reasons below.

Applicant is given two (2) months from the mail date of this Notice within which to respond. This time period for reply is extendable under 37 CFR 1.136(a) for only TWO additional MONTHS.

The informalities requiring correction are indicated in the attachment(s). If the informality pertains to the abstract, specification (including claims) or drawings, the informality must be corrected with an amendment in compliance with 37 CFR 1.121 (or, if the application is a reissue application, 37 CFR 1.173). Such an amendment may be filed after payment of the issue fee if limited to correction of informalities noted herein. See Waiver of 37 CFR 1.312 for Documents Required by the Office of Patent Publication, 1280 Off. Gaz. Patent Office 918 (March 23, 2004). In addition, if the informality is not corrected until after payment of the issue fee, for purposes of 35 U.S.C. 154(b)(1)(iv), "all outstanding requirements" will be considered to have been satisfied when the informality has been corrected. A failure to respond within the above-identified time period will result in the application being ABANDONED.

See attachment(s).

A copy of this notice MUST be returned with the reply. Please address response to "Mail Stop Issue Fee, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450".

/Marty Willis/
Publication Branch
Office of Data Management
(571) 272-4200

Application No. 18352112 **Drawings filed: 07/13/2023**

IDENTIFICATION OF DRAWING DEFICIENCIES

- [ ] There is a hole or the image thereof within the illustration. FIG(s)
- [ ] The illustration is penetrated or traversed by a solid or broken line that is not intended to be part of the drawing, such as a dark line caused by a flaw in the copying process. FIG(s)
- [ ] An ink stamp or the image thereof obscures part of the illustration. FIG(s)
- [ ] The drawing is marred by black smudges, obliterations, or fax/copier marks (for example, speckles or dots in a substantial portion of the drawing). FIG(s)
- [ ] Figure numbers are duplicated or missing. FIG(s)
- [ ] Drawing sheet or figure is missing. FIG(s)
- [ ] Numbers, letters, or reference characters in the drawing have been crossed out or are illegibly handwritten. FIG(s)
- [ ] The character of the lines, numbers, and letters is poor. FIG(s)
- [ ] The drawing's background shows that the original drawing was made on graph paper or other paper with a pattern or decoration. FIG(s)
- [ ] The FIG. number label is placed in a location that causes the drawing to be read upside down. FIG(s)
- [X] Data, a reference number, or part of the drawing is truncated or missing, or a lead line has no reference number. FIG(s) 5
- [ ] The drawing and/or the FIG. label contain(s) foreign language. FIG(s)
- [ ] This utility application contains a photograph of a view that is capable of being illustrated as a line drawing. FIG(s)
- [ ] A petition under 37 CFR 1.84(a)(2) to accept color drawings has been granted, but the brief description of the drawings in the specification does not contain (or has not been amended to contain) the paragraph required by 37 CFR 1.84(a)(2)(iii).
- [ ] This reissue application contains added and/or amended drawings that are not labeled as “New” or “Amended” or “Canceled” as required by 37 CFR 1.173(b)(3). FIG(s)
- [ ] This Design reissue application contains a drawing that is labeled as “Canceled” but is not surrounded by brackets, or a drawing that is surrounded by brackets but is not labeled as “Canceled.” See 37 CFR 1.173(b)(3). FIG(s)
- [ ] OTHER:
- [X] COMMENTS:
Figure 5, on sheet 5/8 dated 7-13-23, contains two reference numbers 501 and 507 that are missing their corresponding lead lines.

TO: docketing@sheppardmullin.com,SheppardMullin_Pair@firsttofile.com
FROM: noreply@uspto.gov
CC: patentcenter_eofficeaction@uspto.gov
SUBJECT: USPTO: Patent Electronic System - Correspondence Notification for Customer Number 30764

Wed Oct 30 05:01:09 EDT 2024

Dear Patent Center Customer:

Correspondence Address:

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650 Town Center Drive, 10th Floor
Costa Mesa,CALIFORNIA,92626
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Application	Document	Mailroom Date	Attorney Docket No.
18352112	M327	10/30/2024	88DG-373535-US

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/352,112	07/13/2023	Emil P. KARTALOV	88DG-373535-US	3145

30764 7590 09/24/2024

Sheppard, Mullin, Richter & Hampton LLP
650 Town Center Drive, 10th Floor
Costa Mesa, CA 92626

EXAMINER	
NEGIN, RUSSELL SCOTT	

ART UNIT	PAPER NUMBER
1672	

NOTIFICATION DATE	DELIVERY MODE
09/24/2024	ELECTRONIC

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docketing@sheppardmullin.com

Response to Rule 312 Communication	**Application No.** 18/352,112	**Applicant(s)**
	Examiner	**Art Unit**

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

1. ☒ The amendment filed on *19 September 2024* under 37 CFR 1.312 has been considered, and has been:

   a) ☒ entered.

   b) ☐ entered as directed to matters of form not affecting the scope of the invention.

   c) ☐ disapproved because the amendment was filed after the payment of the issue fee.
   Any amendment filed after the date the issue fee is paid must be accompanied by a petition under 37 CFR 1.313(c)(1) and the required fee to withdraw the application from issue.

   d) ☐ disapproved. See explanation below.

   e) ☐ entered in part. See explanation below.

Cindy Hilliard

Publishing Division

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California Institute of Technology - Ex. 2026, Page 286

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via the USPTO patent electronic filing system.

By mail, send to: Mail Stop ISSUE FEE
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CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

30764 7590 07/08/2024
Sheppard, Mullin, Richter & Hampton LLP
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Costa Mesa, CA 92626

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(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/352,112	07/13/2023	Emil P. KARTALOV	88DG-373535-US	3145

TITLE OF INVENTION: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	$480	$0.00	$0.00	$480	10/08/2024

EXAMINER	ART UNIT	CLASS-SUBCLASS
NEGIN, RUSSELL SCOTT	1672	702-019000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/AIA/122 or PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/AIA/47 or PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
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1 Sheppard Mullin Richter & Hampton LLP
2 12275 El Camino Real, Suite 100
3 San Diego, CA 92130

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: California Institute of Technology
(B) RESIDENCE: (CITY and STATE OR COUNTRY): Pasadena, CA

Please check the appropriate assignee category or categories (will not be printed on the patent) : ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. Fees submitted: ☒ Issue Fee ☐ Publication Fee (if required)

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

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☒ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. 50-4561

5. **Change in Entity Status** (from status indicated above)

☐ Applicant certifying micro entity status. See 37 CFR 1.29
NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

☐ Applicant asserting small entity status. See 37 CFR 1.27
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

☐ Applicant changing to regular undiscounted fee status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature /David E. Heisey/ Date September 19, 2024

Typed or printed name David E. Heisey Registration No. 42651

PTOL-85 Part B (11/23) Approved for use through 03/31/2026 OMB 0651-0033 U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/352,112	**09/19/2024 08:30:40 PM Z ET**	**88DG-373535-US**

Title of Invention

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	3145	FILED BY	Annie Wong
PATENT CENTER #	67249069	FILING DATE	07/13/2023
CUSTOMER #	30764	FIRST NAMED INVENTOR	Emil P. KARTALOV
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	David Heisey

Documents

TOTAL DOCUMENTS: 1

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
IF_Transmittal_18352112.pdf	1	Issue Fee Payment (PTO-85B)	201 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
IF_Transmittal_18352112.pdf	B5B9C68F75ED6647EEDA964BB8E34C59A0C0BD99B83173E8 E22B0E1265E37F76982219FB377F6BFE717E661974721742C5 B47A86B69B66B29A92F0C0F537A7B1

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

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

New International Application Filed with the USPTO as a Receiving Office

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

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ELECTRONIC PAYMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/352,112	**09/19/2024 08:30:40 PM Z ET**	**88DG-373535-US**

Title of Invention

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	3145	FILED BY	Annie Wong
PATENT CENTER #	67249069	AUTHORIZED BY	David Heisey
CUSTOMER #	30764	FILING DATE	07/13/2023
CORRESPONDENCE ADDRESS	-	FIRST NAMED INVENTOR	Emil P. KARTALOV

Payment Information

PAYMENT METHOD	PAYMENT TRANSACTION ID	PAYMENT AUTHORIZED BY
DA / 504561	**E20249IK31138988**	**Annie Wong**

FEE CODE	DESCRIPTION	ITEM PRICE($)	QUANTITY	ITEM TOTAL($)
2501	UTILITY ISSUE FEE	480.00	1	480.00
			TOTAL AMOUNT:	**$480.00**

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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 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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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/352,112	07/13/2023	Emil P. KARTALOV	88DG-373535-US	3145

30764 7590 08/01/2024

Sheppard, Mullin, Richter & Hampton LLP
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Costa Mesa, CA 92626

EXAMINER
NEGIN, RUSSELL SCOTT

ART UNIT	PAPER NUMBER
1672	

NOTIFICATION DATE	DELIVERY MODE
08/01/2024	ELECTRONIC

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Application No. : 18352112
Applicant : Kartalov
Filing Date : 07/13/2023
Date Mailed : 08/01/2024

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Notice of Allowance Mailed

This application has been accorded an Allowance Date and is being prepared for issuance. The application, however, is incomplete for the reasons below.

Applicant is given two (2) months from the mail date of this Notice within which to respond. This time period for reply is extendable under 37 CFR 1.136(a) for only TWO additional MONTHS.

The informalities requiring correction are indicated in the attachment(s). If the informality pertains to the abstract, specification (including claims) or drawings, the informality must be corrected with an amendment in compliance with 37 CFR 1.121 (or, if the application is a reissue application, 37 CFR 1.173). Such an amendment may be filed after payment of the issue fee if limited to correction of informalities noted herein. See Waiver of 37 CFR 1.312 for Documents Required by the Office of Patent Publication, 1280 Off. Gaz. Patent Office 918 (March 23, 2004). In addition, if the informality is not corrected until after payment of the issue fee, for purposes of 35 U.S.C. 154(b)(1)(iv), "all outstanding requirements" will be considered to have been satisfied when the informality has been corrected. A failure to respond within the above-identified time period will result in the application being ABANDONED.

See attachment(s).

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/Quang Nguyen/
Publication Branch
Office of Data Management
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Application No. 18352112

NON-COMPLIANT ILLUSTRATIONS IN SPECIFICATION OR CLAIMS

[X] **The illustration(s) in the location(s) referenced below do not come within the exceptions of 37 CFR 1.58(a). Please delete the illustration(s) from the specification or claims, provided the illustration(s) as part of the formal drawing(s) in accordance with 37 CFR 1.84, and amend the specification to provide a brief description of the drawings in accordance with 37 CFR 1.74. Please see the following item(s):**

- [X] Specification document coded SPEC dated 07/13/2023, page(s) 90-91.
- [ ] Claims document coded dated , claim(s) .
- [ ] Amendment document coded dated .
- [ ] Other: .

[X] **The chemical formula(s), mathematical formula(s), or table(s) in the location(s) referenced below contain shading (color/grayscale) and therefore do not comply with 37 CFR 1.52 and do not come within the exceptions of 37 CFR 1.58(a). Please delete chemical formula(s), mathematical formula(s), or table(s) with shading from the specification or claims, provide them as formal color drawing(s) in accordance with 37 CFR 1.84, including petition, fee, and amendment in accordance with 37 CFR 1.84(a)(2), and amend the specification to provide a brief description of the drawings in accordance with 37 CFR 1.74. Please see the following item(s):**

- [X] Specification document coded SPEC dated 07/13/2023, page(s) 76-89.
- [ ] Claims document coded dated , claim(s) .
- [ ] Amendment document coded dated .
- [ ] Other: .

SUBSTITUTE SPECIFICATION (clean)

Docket No.: 88DG-373535-US
CIT 6088-C-C-C3-C2

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of U.S. Application No. 16/937,464, filed on July 23, 2020, which is a continuation of U.S. Application No. 15/914,356, filed on March 7, 2018, issued as U.S. Patent No. 10,770,170, which is a continuation of U.S. Application No. 14/451,876, filed on August 5, 2014, issued as U.S. Patent No. 10,068,051, which is a continuation of U.S. Application No. 13/756,760, filed on February 1, 2013, issued as U.S. Patent No. 8,838,394, which claims the benefit of U.S. provisional applications 61/594,480, filed February 3, 2012 and 61/703,093, filed September 19, 2012, each of which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

[0002] This application contains a Sequence Listing which has been submitted electronically in XML format and is incorporated by reference in its entirety. The XML copy, created on July 12, 2023, is named 373535.xml and is 57,570 bytes in size.

GOVERNMENT RIGHTS

[0003] This invention was made with government support under Grant No. 1144469 awarded by the National Science Foundation. The government has certain rights in the invention.

BACKGROUND

[0004] Multiplexed reactions offer significant advantages over traditional uniplex reactions, including performance of parallel reactions on the same sample, use of the same chamber to perform multiple reactions, and the ability to extract rich information from a sample in a fast and efficient manner. However, to achieve these benefits, multiplexed assays generally require complex reporting mechanisms, namely spectrally resolved fluorescence or chemiluminescence (e.g., PCR, ELISA), spatially resolved signals (e.g., microarrays, gel electrophoresis), temporally resolved signals (e.g., capillary electrophoresis), or combinations thereof (e.g., Sanger

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sequencing). There is a need for multiplexed reactions that can be carried out in a single solution.

SUMMARY OF THE INVENTION

[0005] This disclosure provides methods, compositions, systems, and kits for the multiplexed detection of analytes. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some examples, each of the analytes is encoded as a value of one (or at least one) component of a signal.

[0006] In some examples, an assay provided in this disclosure is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = \log_2 (F + 1)$ and F is the maximum cumulative value of one component of a signal (e.g., an intensity) when all the analytes are present.

[0007] In some cases, each analyte is encoded as at least one first value in a first component of a signal (e.g., at least one intensity or range of intensities) and at least one second value in a second component of a signal (e.g., at least one wavelength or range of wavelengths). In some examples, each analyte is encoded as a first value in a first component of a signal at each of a plurality of second values in a second component of a signal (e.g., a signal intensity or range of signal intensities at each of a plurality of wavelengths or ranges of wavelengths).

[0008] In some examples, an assay provided herein is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = C * \log_2 (F + 1)$, where C is the number of the second values used to encode the analytes and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present.

[0009] In some cases, an assay provided in this disclosure is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = (P * T) + 1$, where P is the number of codes per tier in a coding scheme and T is the number of tiers. In some cases, the number of tiers $T = \log_4 (F + 1)$, and F is the maximum cumulative value of a first component of a signal, for any second value, when all of the analytes are present.

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[0010] In some examples, the first value is an intensity or range of intensities. In some cases, a coding scheme comprises at least three intensities or ranges of intensities.

[0011] In some cases, the second value is a wavelength or range of wavelengths. In some examples, the coding scheme comprises at least five wavelengths or ranges of wavelengths.

[0012] In some examples, the signal is an electromagnetic signal. In some cases, the electromagnetic signal is a fluorescence emission signal. In some examples, the intensity of the fluorescence emission signal is measured at at least four wavelengths or ranges of wavelengths.

[0013] In some cases, an assay provided herein is performed with reagents that are lyophilized prior to use.

[0014] In some cases, a detecting step is performed with reagents comprising hybridization probes. In some examples, the number of the hybridization probes is greater than the number of analytes. In some cases, the set of hybridization probes comprise one or more hybridization probes specific for different analytes and comprising an identical fluorophore or combination of fluorophores. In some examples, a sample is contacted with at least 18 of said hybridization probes. In some examples, a detecting step is performed with reagents comprising at least one pair of primers. In some cases, at least one pair of primers are capable of amplifying a region complementary to at least three of said hybridization probes.

[0015] In some examples, the signal that is measured is generated during a polymerase chain reaction. In some cases, the polymerase chain reaction is selected from the group consisting of an end-point polymerase chain reaction, a real-time polymerase chain reaction, a digital polymerase chain reaction, and combinations thereof.

[0016] In some examples, at least one cumulative measurement is performed on a solution.

[0017] In some examples, at least one analyte is encoded by at least one additional value (i.e., at least two values together), wherein the at least one additional value is selected from the group consisting of a value from at least one additional component of a signal, a value from at least one component of a different signal, and combinations thereof. For example, the at least one additional value may be selected from the group consisting of a fluorescence emission intensity, a fluorescence emission wavelength, a Förster resonance energy transfer (FRET) emission intensity, a FRET emission wavelength, an electrochemical signal, a chemiluminescence

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wavelength, a chemiluminescence intensity, a fluorescence bleaching rate, a chemiluminescence bleaching rate, and combinations thereof.

[0018] In some cases, a chromatogram is constructed. The chromatogram may be constructed by plotting all possible combinations of first values and second values for positive control samples for each analyte.

[0019] In some examples, at least one of the analytes comprises a polynucleotide. In some cases, the polynucleotide is from a source selected from the group consisting of an animal, a plant, a bacteria, a fungus, a parasite, and a virus. In some examples, the polynucleotide is from a source selected from the group consisting of human immunodeficiency virus, herpes simplex virus, human papilloma virus, Plasmodium, Mycobacterium, dengue virus, hepatitis virus, and influenza virus. In some cases, the polynucleotide is selected from the group consisting of human immunodeficiency virus polyprotease, human immunodeficiency virus p17, human papilloma virus E6, and human papilloma virus E7.

[0020] In some cases, a sample is selected from the group consisting of a clinical sample, a food sample, an environmental sample, a pharmaceutical sample, and a sample from a consumer product.

[0021] In some examples, information concerning the presence or absence of an analyte is transmitted through a computer network.

[0022] In some cases, information concerning the presence or absence of an analyte is provided to a physician. In some examples, a clinical decision is made based on such information.

[0023] In some examples, at least one step of a method provided herein is performed using instructions on a computer readable medium. In some cases, the instructions are located on a remote server. In some examples, the instructions are located on a thermal cycler. In some cases, the instructions are located on a computer in communication with a thermal cycler.

[0024] In some cases, at least one of the analytes is a positive control analyte.

[0025] In some examples, the coding scheme is non-degenerate. In some cases, the coding scheme is designed to be non-degenerate. In some examples, the coding scheme is made non-degenerate by enumerating every legitimate result that can be obtained from the coding scheme,

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identifying each legitimate result that is degenerate, and eliminating at least one potential analyte code from the coding scheme to eliminate degeneracy.

[0026] In some cases, an assay provided herein is an end-point assay. In some examples an assay provided in this disclosure is ended at a threshold number of cycles set by the limit of detection of an instrument.

[0027] In certain examples, an assay provided herein is a liquid phase assay.

[0028] In some cases, an assay provided herein is quantitative.

[0029] In some cases, this disclosure provides a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

[0030] In some examples, the method described in the preceding paragraph is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = \log_2 (F + 1)$ and F is the maximum cumulative value of the first values when all of the analytes are present. In some cases, the first value is an intensity or range of intensities. In some examples, the first value has a minimum value in the coding scheme that is selected from the group consisting of at least 1, at least 2, at least 4, at least 8, at least 16, at least 32, and at least 64. In some cases, the first value is incremented in the coding scheme by an amount equal to the cumulative maximum of said preceding first values plus one. In other cases, the first value is incremented in the coding scheme by an amount greater than the cumulative maximum of the preceding first values plus one.

[0031] In some cases, this disclosure provides a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as at least

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one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said at least one first value and at least one said second value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme, wherein, when degeneracy is eliminated, said method is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

[0032] In some cases, each of the analytes of the method described in the preceding paragraph is encoded as a first value in a first component of the signal at each of a plurality of second values in a second component of the signal. In some cases, when degeneracy is eliminated, this method is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single volume using four second values. In some examples, when degeneracy is eliminated, the coding scheme comprises T non-degenerate tiers, wherein $T = \log_4 (F + 1)$ and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present. In some cases, when degeneracy is eliminated, the method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = (P * T) + 1$ and P is the number of codes per tier. In some examples, when degeneracy is eliminated, the method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = C * \log_2 (F + 1)$, C is the number of second values in the coding scheme, and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present.

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[0033] This disclosure also provides non-degenerate coding schemes capable of unambiguously encoding the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis.

[0034] In some examples, a non-degenerate coding scheme of this disclosure is generated by a method comprising: (a) generating a code for each potential analyte, wherein each potential analyte is encoded by at least one value of at least one component of a signal; (b) enumerating every legitimate cumulative result for all possible combinations of presence or absence of each analyte; (c) identifying each legitimate result that is degenerate; and (d) eliminating at least one code to eliminate degeneracy. In some cases, the coding scheme may be expanded by adding additional codes that are at least one unit greater than the sum of all previous codes in the at least one component of said signal. In some cases a coding scheme is generated by a method of mathematical iteration that guarantees non-degeneracy by construction.

[0035] In some cases, this disclosure provides systems for detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

[0036] In some cases, this disclosure provides systems for detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding

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is performed in a manner that reduces or eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme, wherein, when degeneracy is eliminated, said system is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

[0037] In some cases, this disclosure provides methods of detecting the presence or absence of each analyte of a plurality of analytes for a third party, comprising: (a) obtaining the identity of each of said analytes from a party; (b) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (c) providing said party with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present, said party: (i) contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and (ii) cumulatively measuring said first values within said sample, thereby providing a cumulative measurement; and (d) obtaining said cumulative measurement from said party; (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and (f) providing said party with information about the presence or absence of each of said analytes.

[0038] In some cases, this disclosure provides methods of detecting the presence or absence of each analyte of a plurality of analytes for a third party, comprising: (a) obtaining the identity of each of said analytes from a party; (b) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme

by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (c) providing said party with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present, said party: (i) contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and (ii) cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and (d) obtaining said cumulative measurement from said party; (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and (f) providing said party with information about the presence or absence of each of said analytes.

[0039] In some cases, this disclosure provides compositions for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising a first value, wherein said reagents are capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

[0040] In some cases, this disclosure provides compositions for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said reagents are capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

[0041] In some cases, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising a first value, wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

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[0042] In some examples, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

[0043] In some cases, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising a kit body (601), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (602), a bottle comprising primers for amplification (603), and a bottle comprising reagents for amplification (604), wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

[0044] In some examples, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, a kit body (601), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (602), a bottle comprising primers for amplification (603), and a bottle comprising reagents for amplification (604), each probe generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said probes generates only one second value.

INCORPORATION BY REFERENCE

[0045] 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.

BRIEF DESCRIPTION OF THE DRAWINGS

[0046] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0047] Fig. 1 shows a comparison between a traditional encoding method of detecting four analytes with four colors and an encoding method of the invention able to detect 16 or more sequences with four colors. Colors are indicated by B, G, Y, and R, which indicate blue, green, yellow, and red, respectively.

[0048] Fig. 2 shows a schematic representation of detection of six analytes (including control) with four colors. The analytes are detected using hybridization probes attached to a fluorophore (B, G, Y, R; blue, green, yellow, and red, respectively) and a quencher (oval with "X"). The control sequence and the five other sequences (Dengue Fever = dengue virus; Tuberculosis = *Mycobacterium tuberculosis*; P17 = HIV p17; Malaria = *Plasmodium falciparum;* and Herpes = herpes simplex virus 2) are all detected using a probe labeled with a blue fluorophore. The non-control analytes are each detected using 1-3 additional probes. For example, the dengue virus analyte is detected using three additional probes with green, yellow, and red fluorophores; the herpes simplex virus 2 analyte is detected using one additional probe with a red fluorophore; and so on.

[0049] Fig. 3 shows chromatograms of experimental results obtained as described in Example 2.

[0050] Fig. 4 shows an exemplary embodiment of the invention in which a computer is used to perform one or more steps of the methods provided herein.

[0051] Fig. 5 shows a schematic of two different methods of detecting wavelength and intensity of a light signal, such as a fluorescence emission signal.

[0052] Fig. 6 shows a schematic of exemplary kits.

[0053] Fig. 7 shows chromatograms of experimental results obtained as described in Example 3.

[0054] Fig. 8 shows a schematic of a legitimate result (top) and an illegitimate result (bottom).

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DETAILED DESCRIPTION OF THE INVENTION

[0055] Fluorescence detection has been a preferred technique for multiplexed assays because of several desirable features, including compatibility with biochemical assays, the relatively small size of fluorescent labels, simple means of conjugation to molecules of interest, affordability, low toxicity, stability, robustness, detectability with inexpensive optics, and an ability to be combined with spatial arrays. However, standard fluorophores have wide emission spectra. Therefore, in order to avoid spectral overlap (i.e., to preserve spectral resolution) only a relatively small number of colors (e.g., 4 to 6) are typically used simultaneously in multiplexed fluorescent assays.

[0056] The traditional encoding method for multiplexed fluorescent assays has been to encode each analyte with a single color; i.e., M=N, where M is the number of analytes that can be detected and N is the number of spectrally resolved fluorescent probes. Whenever higher factors of multiplexing are required (i.e., M>N), fluorescence is generally combined with other techniques, such as aliquoting, spatial arraying, or sequential processing. These additional processing steps are labor-intensive and frequently require relatively expensive and complex optical and mechanical systems (e.g., spectrometers, mechanized microscopy stages, microfluidics, droplet generators, scanners, and the like). Such systems are often impractical to deploy in certain settings, particularly point-of care and low-resource settings. Thus, there is a significant need for multiplexed encoding and decoding methods that can provide an inexpensive means of multiplexing while avoiding the use of expensive additional processing steps.

[0057] This disclosure provides methods, systems, compositions, and kits for the detection of multiple analytes in a sample. Analytes are detected based on the encoding, analysis, and decoding methods presented herein. In some examples, each analyte to be detected is encoded as a value of a signal (e.g., intensity), where the values are assigned so that the results of the assay unambiguously indicate the presence or absence of the analytes being assayed. In other examples, each analyte to be detected is encoded as a value in each of at least two components of a signal (e.g., intensity and wavelength). The at least two components of a signal may be orthogonal. Similarly, as described more fully elsewhere in this disclosure, multiple orthogonal signals may be used, such as a combination of a fluorescent signal and an electrochemical signal.

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The analyte may be any suitable analyte, such as a polynucleotide, a protein, a small molecule, a lipid, a carbohydrate, or mixtures thereof. The signal may be any suitable signal such as an electromagnetic signal, a light signal, a fluorescence emission signal, an electrochemical signal, a chemiluminescent signal, and combinations thereof. The at least two components of the signal may be any suitable two components, such as an amplitude and a frequency or an intensity and a wavelength.

[0058] After encoding of the analytes, a sample is provided wherein the sample comprises or may comprise at least one of the encoded analytes. The sample is contacted with an analyte-specific reagent or reagents that generate a particular signal, as specified for each analyte in the coding scheme, in the presence of an analyte. A reagent may be any suitable reagent that is capable of generating such a signal in the presence of its corresponding analyte, for example, an oligonucleotide probe attached to a fluorophore and a quencher (e.g., a TAQMAN probe). If the reagent is an oligonucleotide probe attached to a fluorophore and a quencher, a nucleic acid amplification may be performed to generate the signal.

[0059] After addition of the reagent(s), the signal is quantified. In some cases, this quantification is performed by measuring one component of the signal (e.g., fluorescence intensity) and determining the presence and absence of certain analytes based on the values used to encode the presence of each analyte and the cumulative value of the signal.

[0060] In some cases, at least two components of a signal (e.g., intensity and wavelength) are cumulatively measured for the sample. This measurement can be performed, for example, by measuring the intensity at a particular wavelength or the intensity within a particular range of wavelengths. The presence or absence of an analyte may then be determined based on the values of each of the at least two components of the signal and the values used to encode the presence of the analyte (i.e., those values in the coding scheme).

[0061] The encoding may be performed in a manner that reduces or eliminates the number of possible degenerate (e.g., ambiguous) results that can be obtained by the method. As described elsewhere in this specification, the full coding capability of a particular coding scheme may be enumerated, and certain potential analyte codes may be eliminated from the coding scheme to reduce or eliminate degeneracy. Similarly, a coding scheme may be designed to be non-

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degenerate, so that a reduction or elimination of degeneracy is not necessary. A decoding matrix may be constructed to translate cumulative signal measurements (e.g., intensities or intensities at particular wavelengths) into the presence or absence of certain analytes, corresponding to the constituent signals of the cumulative signal measurement.

I. Definitions

[0062] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

[0063] As used herein, the singular forms "a," "an," and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms "including," "includes," "having," "has," "with," "such as," or variants thereof, are used in either the specification and/or the claims, such terms are not limiting and are intended to be inclusive in a manner similar to the term "comprising".

[0064] The term "about," as used herein, generally refers to a range that is 15% greater than or less than a stated numerical value within the context of the particular usage. For example, "about 10" would include a range from 8.5 to 11.5.

[0065] The terms "dimension" and "component," as used herein when referring to a signal, generally refer to an aspect of the signal that may be quantified. For example, if a signal is generated by a fluorescent molecule, it may be quantified in terms of its wavelength (e.g., a first dimension or component) and its intensity (e.g., a second dimension or component).

[0066] The term "decoding," as used herein, generally refers to a method of determining which analytes are present based on the cumulative signal and a coding scheme or decoding matrix that enables the conversion of a cumulative signal to information concerning the presence or absence of one or more analytes.

[0067] The term "encoding," as used herein, generally refers to the process of representing an analyte using a code comprising values of a signal, such as intensity, or values in each of at least two components of a signal or signals, such as wavelength and intensity.

[0068] The terms "oligonucleotide probe attached to a fluorophore and a quencher" and "TAQMAN probe" generally refer to hydrolysis probes used to detect the presence of an analyte in a polynucleotide amplification assay. These probes comprise an oligonucleotide probe

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attached to a fluorophore and a quencher. So long as the quencher and the fluorophore are in proximity, the quencher quenches the fluorescence emitted by the fluorophore upon excitation by a light source. The sequence of the oligonucleotide probe is designed to be complementary to a polynucleotide sequence present in an analyte, and therefore capable of hybridizing to the polynucleotide sequence present in the analyte. Hybridization of the oligonucleotide probe is performed in a nucleic acid amplification reaction comprising primers (e.g., a polymerase chain reaction). Upon extension of the primers by a DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and the quencher into the medium. The proximity between the fluorophore and the quencher is broken and the signal from the fluorophore is no longer quenched. Thus, the amount of fluorescence detected is a function of the amount of analyte present. If no analyte is present, the probe will not hybridize to an analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be produced.

[0069] The term "orthogonal," as used herein, generally refers to at least two components of a signal (e.g., wavelength and intensity), or at least two different signals (e.g., fluorescence emission and electrochemical signal), that can be varied independently or approximately independently. For example, wavelength and intensity are considered orthogonal or approximately orthogonal when fluorescent molecules are used. Among other factors, the wavelength of fluorescence emission will depend on the composition of the fluorescent molecule and the intensity of the fluorescence will depend on the amount of molecule present. Although wavelength and intensity are examples of two components of a signal that can be varied approximately independently, the methods described herein are not limited to components that can be varied independently or approximately independently. Components of a signal, or signals, that vary non-independently may also be used, so long as the components or signals are characterized well enough to enable encoding, measurement, and decoding. For example, if the variance in one component or signal affects the variance in another component or signal, the two components or signals may still be used so long as the relationship between the variances is understood.

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[0070] The terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides. Exemplary polynucleotides include deoxyribonucleic acids, ribonucleic acids, and synthetic analogues thereof, including peptide nucleic acids.

[0071] The term "probe," as used herein, generally refers to a reagent capable of generating a signal in the presence of a particular analyte. A probe generally has at least two portions: a portion capable of specifically recognizing an analyte, or a portion thereof, and a portion capable of generating a signal in the presence of an analyte, or a portion thereof. A probe may be an oligonucleotide probe attached to a fluorophore and a quencher, as described above and elsewhere in this disclosure. A probe may also be any reagent that generates a signal in the presence of an analyte, such as an antibody that detects an analyte, with a fluorescent label that emits or is quenched upon binding of the antibody to an analyte. Any suitable probe may be used with the methods presented in this disclosure, so long as the probe generates a quantifiable signal in the presence of an analyte. For example, the analyte-specific portion of a probe may be coupled to an enzyme that, in the presence of an analyte, converts an uncharged substrate into a charged product, thereby increasing the electrical conductivity in the medium over time. In this case, different analytes may be encoded by coupling the analyte-specific portion of the probe (e.g., hybridization probe or antibody) to an enzyme at different ratios. The resulting rate of increased conductivity in the medium will be cumulative for all analytes present in the medium. Encoding analytes according to the methods provided herein enables conversion of the conductivity measurements into unambiguous (i.e., non-degenerate) results providing information about the presence or absence of particular analytes. Similarly, a probe may comprise an enzyme producing a chemiluminescent product from a substrate. The amount of chemiluminescence may then be used to encode the presence of particular analytes.

II. Encoding and Decoding Methods

A. Traditional Fluorescent Encoding and Decoding Method

[0072] A commonly used method of determining the presence of an analyte uses four spectrally resolved fluorescent molecules to indicate the presence or absence of four analytes. An example of this method is presented on the left-hand side of **Fig. 1**. The left-hand side of **Fig. 1** shows an

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encoding method where four analytes (Seq 1, Seq 2, Seq 3, and Seq 4) are each encoded by a single color (blue, green, yellow, and red, respectively). The color represents a fluorophore attached to an oligonucleotide probe that also comprises a quencher. In the system shown on the left-hand side of **Fig. 1**, there are four different oligonucleotide probes, each comprising a single fluorophore (blue, green, yellow, or red) and a quencher. The presence or absence of an analyte is determined based on the presence or absence of a signal in a particular color.

[0073] The chart on the left-hand side of **Fig. 1** shows intensity versus color for a hypothetical sample containing two analytes: Seq 1 and Seq 3. The presence of these analytes is determined based on the measurement of a blue signal (corresponding to Seq 1) and a yellow signal (corresponding to Seq 3). The absence of Seq 2 and Seq 4 is indicated by the absence of a blue and red signal.

[0074] Table 1 shows a translation of this coding scheme into a binary format. Each analyte is encoded as a value in each of two components of a fluorescent signal: (1) color (also known as wavelength; or range of wavelengths) and (2) intensity (indicated by the numbers within the table). For example, A (e.g., Seq 1) has a color of blue and an intensity of 1; B (e.g., Seq 2) has a color of green and an intensity of 1; and so on. The intensity of the signal within each color range may be quantified as described herein, for example by measuring the intensity of the signal within a particular wavelength range determined by a band pass filter. A result of 1000 indicates that only analyte A is present; a result of 1100 indicates that analytes A and B are present; and so on.

Table 1. Traditional encoding of four analytes with four probes, each probe having a single color, and one probe per analyte.

	Blue	**Green**	**Yellow**	**Red**
A	1	0	0	0
B	0	1	0	0
C	0	0	1	0
D	0	0	0	1

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B. Encoding Methods Using More Than One Color Per Analyte

[0075] The traditional method described above suffers from the fact that it is limited by the number of spectrally resolvable fluorophores. More specifically, the number of detectable analytes is equal to the number of spectrally resolvable fluorophores. Therefore, the number of analytes may only be increased by increasing the number of spectrally resolvable fluorophores.

[0076] This disclosure provides methods that overcome this limitation. More specifically, in some cases, by utilizing at least two components of a signal during encoding, the methods described herein may be used to detect more than one analyte per fluorophore. For example, using the method provided herein 1.5, 1.6, 1.7, 1.8, 1.9, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes may be detected per fluorophore. In some cases, the methods provided herein may be used to detect at least 1.5, 1.6, 1.7, 1.8, 1.9, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes may be detected per fluorophore. In some cases, the methods provided herein may be used to detect 1.5-2, 2-4, 1.5-4, 4-6, 2-6, 6-10 analytes per fluorophore.

[0077] In some cases, the methods provided in this disclosure may include the use of a control color. The control color may be attached to one or more probes binding a positive control analyte, and each analyte to be detected, in a sample. If the same sequence occurs in the positive control analyte and each analyte to be detected, a single control probe may be used. If the same sequence does not occur in the positive control analyte and each analyte to be detected, different probes may be used, but each probe may still be attached to the control color.

[0078] For example, building on the traditional methods described above, one color (e.g., blue) may be used to encode the presence of a control analyte that is always present in the sample. The control analyte may be added to the sample, or may be inherently present in the sample. The remaining colors (e.g., green, yellow, red) may be used to encode the presence of additional analytes. Table 2 shows an example of one such method.

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Table 2. Encoding of four analytes, including control, with four colors and up to two colors per analyte.

	Blue	Green	Yellow	Red
Ctrl. (p)	1	0	0	0
A	1	0	0	1
B	1	0	1	0
C	1	1	0	0

[0079] In Table 2, the presence of the control (p) is indicated by a result of 1000. The presence of the control and analyte A is indicated by a result of 2001. The presence of the control and all three other analytes is indicated by a result of 4111. The control color (blue) provides an indication that the assay is functioning properly. The intensity of the blue color reports the number of analytes that are present in a test sample. Of course, any color may be used as the control color. One of skill in the art will recognize that certain practical considerations might make it preferable to use one color over another as the control color. For example, if one color is better detected in a particular optical system (or system of fluorophores), it might be practically preferable to use that color as a control color.

[0080] The coding scheme shown in Table 2 encodes each analyte using one control color and one additional color (up to 2 probes per analyte). However, as shown below, the number of analytes that can be encoded increases when up to 4 colors are used per analyte. Table 3 shows an exemplary coding scheme where each analyte is encoded by up to 4 colors. In the scheme shown in Table 3, the control color (blue) is generated in the presence of the control sequence and each of the other seven analytes (A-G). The other seven analytes are each encoded by the presence of one to three additional colors. The colors may be contained on different probes (e.g., oligonucleotide probes attached to a fluorophore and a quencher) or on the same probe (which can have multiple fluorophores and multiple quenchers).

Table 3. Encoding of eight analytes (including control) with four colors and up to four colors per analyte.

	Blue	Green	Yellow	Red
Ctrl. (p)	1	0	0	0
A	1	0	0	1

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B	1	0	1	0
C	1	1	0	0
D	1	0	1	1
E	1	1	0	1
F	1	1	1	0
G	1	1	1	1

[0081] In Table 3, the presence of the control and all seven other analytes is indicated by a result of 8444. Three analytes (A, B, and C) are encoded by two colors. Three analytes (D, E, and F) are encoded by three colors, and one analyte (G) is encoded by four colors. The presence of the control (p) is indicated by a result of 1000. The presence of the control and analyte A is indicated by a result of 2001. The presence of the control and analyte G is indicated by a result of 2111. These results express the cumulative intensity of the signal in each color. For example, the result 2111 has a 2X signal intensity in the blue channel, while the result 1000 has only a 1X signal intensity in the blue channel.

[0082] Using Table 3, each of the possible cumulative assay results can be enumerated, in terms of color (blue, green, yellow, red) and intensity (0-8). Table 4 shows a "decoding matrix" generated by enumerating each of the possible cumulative assay results based on the encoding method presented in Table 3 and providing the corresponding decoded result of each assay in terms of the analyte(s) present in the sample. A similar decoding matrix may be generated for any coding scheme described herein, by enumerating each of the possible cumulative assay results based on the coding scheme and providing the corresponding decoded result of each assay in terms of the analyte(s) present in (or absent from) the sample. In some cases, as described below, one or more analytes may be removed from the coding scheme in order to reduce or eliminate degeneracy.

Table 4. Decoding matrix for encoding method presented in Table 3.

Cumulative Assay Result				
Blue	**Green**	**Yellow**	**Red**	**Analyte(s) Present**
1	0	0	0	p
2	0	0	1	pA
2	0	1	0	pB
2	1	0	0	pC

2	0	1	1	pD
2	1	0	1	pE
2	1	1	0	pF
2	1	1	1	pG
3	0	1	1	pAB
3	1	0	1	pAC
3	0	1	2	pAD
3	1	0	2	pAE
3	1	1	1	pAF, pCD, pBE
3	1	1	2	pAG, pDE
3	1	1	0	pBC
3	0	2	1	pBD
3	1	2	0	pBF
3	1	2	1	pBG, pDF
3	2	0	1	pCE
3	2	1	0	pCF
3	2	1	1	pCG, pEF
3	1	2	2	pDG
3	2	1	2	pEG
3	2	2	1	pFG
4	1	1	1	pACB
4	0	2	2	pABD
4	2	2	0	pBCF
4	2	0	2	pACE
4	1	1	2	pABE, pACD
4	1	2	1	pABF, pBCD
4	2	1	1	pACF, pBCE
4	1	2	2	pADF, pABG, pBDE
4	2	1	2	pACG, pCDE, pAEF
4	2	2	1	pBCG, pBEF, pCDF
4	2	2	2	pCDG, pAFG, pBEG, pDEF
4	1	1	3	pADE
4	1	3	1	pBDF
4	3	1	1	pCEF
4	1	2	3	pADG
4	3	2	1	pCFG
4	2	1	3	pAEG
4	2	3	1	pBFG
4	1	3	2	pBDG
4	3	1	2	pCEG
4	2	2	3	pDEG
4	3	2	2	pEFG

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5	1	2	2	pABCD
5	2	1	2	pABCE
5	2	2	1	pABCF
5	2	2	2	pABCG, pABEF, pBCDE, pACDF
5	1	2	3	pABDE
5	2	1	3	pACDE
5	1	3	2	pABDF
5	2	1	3	pBCDF
5	3	1	2	pACEF
5	3	2	1	pBCEF
5	2	2	3	pABEG, pACDG, pADEF
5	2	3	2	pABFG, pBCDG, pBDEF
5	3	2	2	pACFG, pBCEG, pCDEF
5	1	3	3	pABDG
5	3	1	3	pACEG
5	3	3	1	pBCFG
5	2	3	3	pADFG, pBDEG
5	3	3	2	pBEFG, pDEFG
5	3	2	3	pCDEG, pAEFG
5	2	2	4	pADEG
5	2	4	2	pBDFG
5	4	2	2	pCEFG
5	3	3	3	pDEFG
6	2	2	3	pABCDE
6	2	3	2	pABCDF
6	3	2	2	pABCEF
6	2	3	3	pABCDG, pABDEF
6	3	2	3	pABCEG, pACDEF
6	3	3	2	pABCFG, pBCDEF
6	4	3	2	pBCEFG
6	4	2	3	pACEFG
6	3	3	3	pABEFG, pACDFG, pBCDEG
6	3	4	2	pBCDFG
6	2	4	3	pABDFG
6	3	2	4	pACDEG
6	2	3	4	pABDEG
6	4	3	3	pCDEFG
6	3	4	3	pBDEFG
6	3	3	4	pADEFG
7	4	4	3	pBCDEFG
7	4	3	4	pACDEFG
7	3	4	4	pABDEFG

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7	4	3	3	pABCEFG
7	3	4	3	pABCDFG
7	3	3	4	pABCDEG
7	3	3	3	pABCDEF
8	4	4	4	pABCDEFG

[0083] The decoding matrix provided in Table 4 is constructed using two assumptions. First, the decoding matrix assumes that the positive control (p) always produces a positive outcome. Second, the decoding matrix assumes that, within each color, the intensity is additive and scales in the same way with changing probe concentration, regardless of which probe the signal may come from. This essentially means that the signals are additive and digital. The conditions underlying these assumptions may be met by properly preparing the assay. If a fluorescent signal is used, the intensity need only be approximately additive and digital, as demonstrated in the Examples provided herein.

[0084] Table 4 allows the conversion of a cumulative measurement of intensity in four ranges of fluorescent wavelengths (i.e., signal intensity within each color range), into the corresponding analytes present in the sample. For example, a result of 4321 indicates that p, C, F, and G are present and the other analytes are not present. This result is referred to as a "legitimate" result, because it is present in the decoding matrix. By contrast, a result of 4000, while possible to measure, does not occur in the decoding matrix. More specifically, the result of 4000 cannot be achieved by adding any combination of control and analyte codes from Table 3. This result is referred to as an "illegitimate" result. An illegitimate result may indicate that the assay malfunctioned. Thus, the control (p) and the decoding matrix provide a means of verifying that the assay is functioning properly.

[0085] Table 4 is exhaustive for any combination of fluorophores generating four resolvable emission spectra (e.g., colors). The term "rank" is used to describe the number of detected analytes, including the control. In the example provided above, if the assay functions properly, the rank is equal to the value of the blue signal. For example, a rank of 8 indicates that the control and all seven other analytes (A-G) are present. A rank of 2 indicates that the control and only one analyte are present. The lowest rank is a rank of 1, which indicates that only the control

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is present. The number of possibilities at each rank can be enumerated. For example, continuing to refer to the encoding and decoding method described in Tables 3-4, there are 7 possibilities for rank 2. The number of possibilities at rank 3 can be calculated as a combination of 7 take 2, or 7!/(5! * 2!) = 21 possibilities. More generally, the number of possibilities for a combination of N take K is N!/((N-K)! * K!). Analogously, at ranks 4, 5, 6, 7, and 8, the number of possibilities is 35, 35, 21, 7, and 1, respectively. Referring to Table 4 shows that the table agrees with the theoretical prediction. Thus, Table 4 is an exhaustive decoding matrix for the encoding method provided in Table 3.

C. Reducing or Eliminating Degeneracy

[0086] The terms "degenerate" and "degeneracy," as used herein, generally describe a situation where a legitimate result is not definitive, because it can indicate more than one possibility in terms of the presence or absence of an analyte. For example, with reference to Table 4, result 5233 is degenerate because it can be decoded as either pADFG or pBDEG. Similarly, result 4222 is degenerate because it can be decoded as any of the following: pCDG, pAFG, pBEG, pDEF. By contrast, result 3110 can only indicate pBC and thus is not degenerate.

[0087] This disclosure provides methods of reducing or eliminating degeneracy, thereby increasing the confidence with which an analyte is detected. In one embodiment, degeneracy is eliminated by a method comprising (i) encoding each potential analyte to be detected as a value of a signal and, optionally, as a value in each of at least two components of a signal; (ii) enumerating every legitimate result that can be obtained from the coding scheme; (iii) identifying each legitimate result that is degenerate; and (iv) eliminating at least one potential analyte (or potential analyte code) from the coding scheme, wherein eliminating the at least one potential analyte reduces or eliminates degeneracy. For example, with reference to the coding scheme described in Table 3 and the decoding matrix described in Table 4 (enumerating every legitimate result), eliminating any two of analytes D, E, and F eliminates the degeneracy. Eliminating any one of analytes D, E, and F would not eliminate the degeneracy, but would reduce it.

[0088] With continued reference to the coding scheme described in Table 3, eliminating any two of analytes D, E, and F from the coding scheme results in a scheme where six analytes (including

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control) can be analyzed, with no degeneracy, using only 4 colors. By contrast, conventional methods of multiplexing would allow for only the reporting of 3 analytes and 1 control using 4 colors. Therefore, the number of analytes that can be analyzed is nearly doubled by using the methods provided herein.

[0089] Fig. 2 shows an exemplary embodiment of the invention in which 4 colors are used to detect five analytes and a control. The analytes are nucleic acids from dengue virus ("Dengue Fever"), *Mycobacterium tuberculosis* ("Tuberculosis"), human immunodeficiency virus (HIV) p17 (P17), *Plasmodium* ("Malaria"), and herpes simplex ("Herpes"). The "colors" in this example are fluorophores attached to oligonucleotide probes that also comprise a quencher. The oligonucleotide probes will generally be different for different analytes, whereas the color is the same. For example, all probes designated as Probe 1 have a blue color but generally will have a different oligonucleotide sequence. Of course, probes designated as Probe 1 could also have the same oligonucleotides sequence, if the complementary sequence was present in each of the analytes. The mechanism of detection with these probes is described elsewhere in this disclosure. Probe 1 (blue) hybridizes to all six analytes, including control. Probe 2 (green) hybridizes to analytes from dengue virus, *Mycobacterium*, and HIV p17. Probe 3 (yellow) hybridizes to analytes from dengue fever and *Plasmodium*. Probe 4 hybridizes to analytes from dengue virus, *Mycobacterium*, and herpes simplex. The coding scheme defined by these probes is illustrated in Table 5. With reference to Table 3, potential analytes D (1011) and F (1110) have been eliminated from the coding scheme. Therefore, the coding scheme presented in Table 5 is non-degenerate and each legitimate result from the assay corresponds to the presence or absence of a unique combination of analytes in a sample.

Table 5. Coding scheme for detection of dengue virus, *Mycobacterium*, HIV, *Plasmodium*, and herpes simplex, as exemplified in Fig. 2.

	Blue	**Green**	**Yellow**	**Red**
Ctrl. (p)	1	0	0	0
Herpes Simplex	1	0	0	1
Plasmodium	1	0	1	0
HIV	1	1	0	0

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Mycobacterium	1	1	0	1
Dengue Virus	1	1	1	1

[0090] The methods for encoding and decoding presented above, including the methods for eliminating degeneracy, are all equally applicable to encoding methods using additional colors. For example, Table 3 could be extended by including additional colors and additional intensities (described further below). The decoding matrix is then generated as described above, enumerating every legitimate result that can be obtained from the coding scheme. A decoding matrix analogous to the decoding matrix provided in Table 4 may be constructed for any coding scheme described in this disclosure. The legitimate results that are degenerate are then identified and at least one potential analyte code is eliminated from the coding scheme to reduce or eliminate degeneracy. The method of eliminating degeneracy may be carried out using software on a computer readable medium, or hardware configured to carry out the method (e.g., a microchip).

[0091] Although degeneracy can be reduced or eliminated by the methods described above, and elsewhere in this disclosure, this disclosure also provides coding schemes that are non-degenerate by design. For example, after elimination of degeneracy in the coding scheme described in Table 3, the coding scheme may be extended indefinitely in a non-degenerate manner where the non-degeneracy is by design (see, e.g., the coding scheme exemplified in Table 6, described more fully below). Similarly, this disclosure provides coding schemes that are completely non-degenerate by design and therefore do not require any reduction or elimination of degeneracy (see, e.g., the coding scheme exemplified in Table 8, described more fully below). Thus, the coding schemes provided in this disclosure may have reduced or eliminated degeneracy, or be non-degenerate by design.

D. Encoding Methods Using More Than One Color and More Than One Intensity

[0092] The encoding method described above (e.g., Tables 3-5) may be further extended by allowing analytes to be encoded by an intensity greater than 1. For example, each of the analytes encoded in the coding scheme provided in Table 5 is encoded by a fluorescence intensity of

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either 1 or 0. Allowing higher values for the signal intensity in at least one color further increases the number of analytes that can be encoded by any of the methods provided in this disclosure. In some examples, these higher intensity values may be assigned any color except for the control color, in order to maintain the analyte counting capability of the control color.

[0093] Table 6 shows the first three tiers of an exemplary coding scheme that utilizes four colors and multiple intensities. Tier 1 of Table 6 is a reproduction of Table 3, showing the encoding of seven analytes and a control with four colors. As described above, any two of potential analytes D, E, and F may be eliminated from the coding scheme in order to produce a non-degenerate coding scheme. Tier 1 of Table 6 indicates that potential analytes D (1011) and F (1110) have been eliminated from the coding scheme to eliminate degeneracy. Therefore, the coding scheme presented in Tier 1 of Table 6 is capable of determining the presence of five analytes and one control using four colors, as described above.

[0094] The coding scheme of Tier 1 of Table 6 may be expanded to a second tier (Tier 2) by allowing the intensity in any of the colors to increase. As described above, the intensity of the control color may be maintained at 1, in order to preserve the sequence counting capability of the control. Increasing the intensity of any of the remaining three colors will yield codes 100Y, 10Y0, 1Y00, 10YY, 1Y0Y, 1YY0, and 1YYY, where $Y > 1$. The minimal value of Y for a new tier of encoding is equal to the cumulative maximum value from the prior tier(s) plus 1. As described below, a value greater than 1 could also be used, to maximize the differences between the intensities.

[0095] Thus, in this context, the term "tier" is generally used to describe a set of codes that fully utilize the coding capability provided by a particular number of first values (e.g., intensities) and second values (e.g., colors), without degeneracy. For example, Tier 1 of Table 6 fully utilizes the coding capability provided by four colors with up to one intensity in each color, without degeneracy. As shown in Table 6, Tier 1, this results in six encoded analytes, including the control. To introduce a second tier, the minimum value of Y (described above) may be incremented to equal the cumulative maximum result from the prior tier(s) plus one (or more than one). In the example provided in Table 6, the intensity of the blue (control) color is maintained as one, to preserve the sequence counting capability in this color. Thus, Tier 2

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consists of five non-degenerate encoding possibilities obtained by incrementing the intensities in the green, yellow, and red channels to equal the cumulative maximum results in each of these channels from Tier 1, plus one. All possibilities of these codes may then be enumerated for Tier 2, and codes resulting in degeneracy may be eliminated, or Tier 2 may be made non-degenerate by design, using the information used to eliminate the corresponding codes from Tier 1. Further coding capacity may then be achieved by adding a third tier, or further tiers, which are constructed according to analogous methods. A coding scheme may have an infinite number of tiers, for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100 or more tiers.

[0096] More specifically, with reference to Table 6, Tier 2, analyte H is encoded by 1004. The value of the control color is maintained as 1. The value of the red color is equal to the cumulative maximum result from the prior tier (3) plus 1, or 4. Similarly, analytes I and J are encoded by 1030 and 1400, respectively. Combinations of these codes are used to encode the remaining four analytes (K-N), as was done for analytes D-G in Tier 1. This completes Tier 2. In Tier 2, the inclusion of potential analytes K (1034) and M (1430) results in degeneracy. Therefore, these analytes have been eliminated from the coding scheme, to eliminate degeneracy.

[0097] Continuing to refer to Table 6, a third tier (Tier 3) is constructed using the same principles described above. Analyte O is encoded by 1-0-0-16. The value of the control color is still maintained as 1. The value of the red color is equal to the cumulative maximum result from the prior tiers (15) plus 1, or 16. Similarly, analytes P and Q are encoded by 1-0-9-0 and 1-16-0-0, respectively. Combinations of these codes are used to encode the remaining four analytes (R-U), as was done for analytes D-G in Tier 1 and analytes K-N in Tier 2. In Tier 3, the inclusion of potential analytes R (1-0-9-16) and T (1-16-9-0) results in degeneracy. Therefore, these analytes have been eliminated from the coding scheme, to eliminate degeneracy.

[0098] The three-tier coding scheme shown in Table 6 shows the encoding of 15 analytes and one control using four colors. This coding scheme may be indefinitely extended, by adding more intensities to generate additional tiers and/or adding more colors, to generate additional coding capability within the tiers. The methods of reducing or eliminating degeneracy, as described in

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this disclosure, may be used with the addition of each intensity and/or color, to reduce or eliminate degenerate results.

[0099] More generally, the coding scheme depicted in Table 6 is a non-degenerate, infinite extension of the coding scheme depicted in Tables 3-5. The maximum intensity of the cumulative measurement at the first tier is 6. The maximum intensity of the cumulative measurement at the second tier is 15. The maximum intensity of the cumulative measurement at the third tier is 63, and so on. Given a maximum cumulative intensity value (F), the maximum number of tiers (T) available in this coding scheme is $T = \log_4 (F + 1)$. The coding scheme depicted in Table 6 provides five non-degenerate codes per tier (P). Thus, the maximum number of codes $M = 5 * \log_4 (F + 1)$, or $M = P * T$, where P is the number of non-degenerate codes per tier and T is the number of tiers. For example, given $F = 63$, the maximum number of codes (i.e., analytes) is 15 for 5 non-degenerate codes per tier. This formula does not include the 1000 code, which is reserved for a positive control in Table 6. To include the control in the total number of analytes, one would simply add one, to provide the formula $M = (P * T) + 1$.

[00100] The methods provided in this disclosure may be used to expand this coding scheme infinitely. For example, by utilizing combinations of different intensities (i.e., first values) and colors (i.e., second values) one can encode any number of analytes (M) by varying the number of non-degenerate codes per tier (P) and the number of tiers (T). For example, the number of non-degenerate codes per tier is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more. Similarly, the number of tiers may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more.

Table 6. Encoding of 15 analytes and one control using four colors and multiple intensities.

Tier	Analyte	B	G	Y	R	Comments
1	**Ctrl. (p)**	1	0	0	0	
	A	1	0	0	1	
	B	1	0	1	0	

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	C	1	1	0	0	
	~~**D**~~	~~1~~	~~0~~	~~1~~	~~1~~	Eliminated from coding scheme to eliminate degeneracy.
	E	1	1	0	1	
	~~**F**~~	~~1~~	~~1~~	~~1~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	G	1	1	1	1	
Cumulative Maximum Result		*6*	*3*	*2*	*3*	
2	**H**	1	0	0	4	
	I	1	0	3	0	
	J	1	4	0	0	
	~~**K**~~	~~1~~	~~0~~	~~3~~	~~4~~	Eliminated from coding scheme to eliminate degeneracy.
	L	1	4	0	4	
	~~**M**~~	~~1~~	~~4~~	~~3~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	N	1	4	3	4	
Cumulative Maximum Result		*11*	*15*	*8*	*15*	
3	**O**	1	0	0	16	
	P	1	0	9	0	
	Q	1	16	0	0	
	~~**R**~~	~~1~~	~~0~~	~~9~~	~~16~~	Eliminated from coding scheme to eliminate degeneracy.
	S	1	16	0	16	
	~~**T**~~	~~1~~	~~16~~	~~9~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	U	1	16	9	16	
Cumulative Maximum Result						
Cumulative Maximum Result for Three Non-Degenerate Tiers		***16***	***63***	***26***	***63***	

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[00101] The right-hand side of **Fig. 1** shows one exemplary embodiment of the method described above. More specifically, the right-hand side of **Fig. 1** shows an encoding method where nine or more analytes (Seq 1 - Seq 9, etc.) are each encoded by at least two colors, with varying intensities within each color. The color represents a fluorophore attached to an oligonucleotide probe that also comprises a quencher. The system can be designed so that each probe is labeled with single fluorophore, or each probe is labeled with more than one fluorophore. For example, the code for analyte H, in Table 6, is 1004. The intensity of 4 in the red channel may be achieved by either using an H-specific probe comprising 4 red fluorophores, or by using 4 H-specific probes each comprising a single red fluorophore. Of course, any combination of probes and fluorophores producing a result of 4 in the red channel would be equally appropriate, such as 2 probes with 2 red fluorophores each, and 1 probe with 1 red fluorophore and 1 probe with 3 red fluorophores, or simply one probe with one red fluorophore but present at 4x amount in the reaction mixture.

[00102] The coding scheme depicted on the right-hand side of **Fig. 1** may be represented as in Table 7. The result of the analysis shown on the right-hand side of **Fig. 1** is 4112, or an intensity of 4 in the blue channel, 1 in the green channel, 1 in the yellow channel, and 2 in the red channel. Using a decoding matrix constructed as described herein, this result is decoded to indicate the presence of Seq 1, Seq 3, Seq 4, and Seq 5.

Table 7. Coding scheme illustrated on the right-hand side of Fig. 1.

Tier	Analyte	B	G	Y	R	Comments
1	**Seq 1**	1	0	0	0	
	Seq 2	1	1	0	0	
	Seq 3	1	0	1	0	
	Seq 4	1	0	0	1	
	Seq 5	1	1	0	1	
	Seq 6	1	1	1	1	
	~~**Eliminated Potential Seq**~~	~~1~~	~~0~~	~~1~~	~~1~~	Eliminated from coding scheme to eliminate degeneracy.
	~~**Eliminated Potential Seq**~~	~~1~~	~~1~~	~~1~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.

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Cumulative Maximum Result		*6*	*3*	*2*	*3*	
2	**Seq 7**	1	4	0	0	
	Seq 8	1	0	3	0	
	Seq 9	1	0	0	4	
Etc. - i.e. Continue as provided in Table 6.						

E. Encoding Methods Using One Color and One Intensity Per Analyte but Different Intensities Among Analytes

[00103] In some methods provided herein, each analyte is encoded by a single color and intensity combination. For example, in a four color system, the first four analytes may be encoded by 1000, 2000, 4000, and 8000. The next four analytes may be encoded by 0100, 0200, 0400, and 0800. Analytes 9-12 and 13-16 would be assigned analogously, as shown in Table 8.

[00104] Like the encoding method described in Table 6, this coding scheme is theoretically infinite, non-degenerate by construction, and only limited by the bandwidth of the instrument used to measure the signal. However, this coding scheme enables more analytes to be quantified per unit of bandwidth than the encoding method described in Table 6. The reason is that the available multiplicity of signal is used with maximal efficiency, as each level of intensity is utilized in the coding (i.e. there are no gaps in the chromatogram; see below for description of chromatograms). Table 8 shows one embodiment of this method, illustrating four tiers of encoding based on four colors and intensities 1, 2, 4, and 8. The coding scheme is non-degenerate, and the result if all 16 analytes are present is 15-15-15-15. This encoding method is more efficient, in terms of bandwidth utilization, than the encoding methods presented above. However, this method does not have the proofreading capability of the first scheme, as all the results decode to legitimate outcomes in the absence of gaps in the chromatogram.

Table 8. Example of encoding method using one color and one intensity per analyte, but different intensities among analytes.

Tier	Analyte	B	G	Y	R
1	**A**	1	0	0	0
	B	2	0	0	0

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	C	4	0	0	0
	D	8	0	0	0
2	**E**	0	1	0	0
	F	0	2	0	0
	G	0	4	0	0
	H	0	8	0	0
3	**I**	0	0	1	0
	J	0	0	2	0
	K	0	0	4	0
	L	0	0	8	0
4	**M**	0	0	0	1
	N	0	0	0	2
	O	0	0	0	4
	P	0	0	0	8
		15	***15***	***15***	***15***

[00105] The method presented above, and exemplified in Table 8, may be extended by introducing additional colors and/or intensities. For example, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more colors may be used. Additional intensity levels, such as 16, 32, 64, 128, 255, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, and so on may also be used. The method is generalizable and may be used to encode an infinite number of analytes. Each analyte is represented by a code in a single color, wherein the value of the code in that single color equal to the sum of all previous values plus one. For example, if the first code contains a 1 in a particular color, the next codes are 2, 4, 8, 16, 32, 64, 128 and so on. Other progressions are possible, but will not be as efficient in terms of usage of the bandwidth. However, one of ordinary skill in the art will recognize that less efficient use of bandwidth may be desirable when one wishes to maximize separation between values. Similarly, the value 1 could be excluded from the coding scheme, for example to maximize the difference in the intensity between the first encoded value and instrumental noise. For example, in some cases a

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coding scheme may begin with a value of 2, which would provide a progression of 2, 4, 8, 16, 32, 64, 128, and so on for the analyte codes, while the progression of cumulative results would be 2, 4, 6, 8, 10, 12, 14, 16, and so on. This approach generates uniform gaps in the possible cumulative results, allowing for higher tolerance to noise in comparison to an analyte code progression of 1, 2, 4, 8, 16, 32, and so on, and its corresponding cumulative result progression of 1, 2, 3, 4, 5, 6, 7, 8, and so on. In this example, the increased robustness in the measurement comes at a cost of a decrease in the number of available codes within a fixed bandwidth of multiplicity. For example, if only 63 multiplicity states can be measured reliably, the coding scheme starting with 1 offers 7 codes per color whereas the coding scheme starting with 2 offers 6 codes per color. If 4 colors are available, the former will offer 28 codes, while the latter will offer 24 codes. In summary, both the starting signal intensity and the progression (i.e., difference between intensity values) may be scaled in order to maximize the ability to distinguish over instrumental noise and maximize the differences between the intensity values, thereby enhancing the ability to distinguish between distinct experimental outcomes.

[00106] The coding scheme illustrated in Table 8 is non-degenerate by design. Although the coding scheme illustrated in Table 8 uses both intensity and color to encode each of the 16 analytes, each of the analytes could also be encoded by simply utilizing intensity. For example, given the 16 analytes provided in Table 8 (A-P), a single color coding scheme encoding all 16 analytes may assign the values 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, and 16384 to analytes A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, and P, respectively. Such a coding scheme is non-degenerate by design and the cumulative intensity result can be unambiguously decoded to indicate the presence or absence of analytes A-P. This coding scheme is capable of detecting the presence or absence of M analytes, where $M = \log_2 (F + 1)$ and F is the maximum cumulative value of a signal (e.g., signal intensity). By adding a second component to this signal (e.g., color, as depicted in Table 8), the capacity of this coding scheme can be increased to $M = C * \log_2 (F + 1)$, where C is the number of colors used in said coding scheme.

[00107] As described throughout this specification, any suitable value may be used for C or F. For example, C may be at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44,

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45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more. *F* may be at least about 2, 4, 8, 16, 32, 64, 128, 255, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, 131072, 262144, 524288, 1048576, and so on. As described elsewhere in this disclosure, both the initial starting value of *F* and the steps in its progression may also be varied.

III. Analytes

[00108] An analyte may be any suitable analyte that may be analyzed using the methods and compositions of the invention, where the analyte is capable of interacting with a reagent in order to generate a signal with at least two components that can be measured. An analyte may be naturally-occurring or synthetic. An analyte may be present in a sample obtained using any methods known in the art. In some cases, a sample may be processed before analyzing it for an analyte. The methods and compositions presented in this disclosure may be used in solution phase assays, without the need for particles (such as beads) or a solid support.

[00109] In some cases, an analyte may be a polynucleotide, such as DNA, RNA, peptide nucleic acids, and any hybrid thereof, where the polynucleotide contains any combination of deoxyribo- and/or ribo-nucleotides. Polynucleotides may be single stranded or double stranded, or contain portions of both double stranded or single stranded sequence. Polynucleotides may contain any combination of nucleotides or bases, including, for example, uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine, isoguanine and any nucleotide derivative thereof. As used herein, the term "nucleotide" may include nucleotides and nucleosides, as well as nucleoside and nucleotide analogs, and modified nucleotides, including both synthetic and naturally occurring species. Polynucleotides may be any suitable polynucleotide for which one or more reagents (or probes) as described herein may be produced, including but not limited to cDNA, mitochondrial DNA (mtDNA), messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), nuclear RNA (nRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small Cajal body-specific RNA (scaRNA), microRNA (miRNA), double stranded (dsRNA), ribozyme, riboswitch or viral RNA. Polynucleotides may be contained within any suitable vector, such as a plasmid, cosmid, fragment, chromosome, or genome.

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[00110] Genomic DNA may be obtained from naturally occurring or genetically modified organisms or from artificially or synthetically created genomes. Analytes comprising genomic DNA may be obtained from any source and using any methods known in the art. For example, genomic DNA may be isolated with or without amplification. Amplification may include PCR amplification, multiple displacement amplification (MDA), rolling circle amplification and other amplification methods. Genomic DNA may also be obtained by cloning or recombinant methods, such as those involving plasmids and artificial chromosomes or other conventional methods (see Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*., cited *supra*.) Polynucleotides may be isolated using other methods known in the art, for example as disclosed in *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV) or *Molecular Cloning: A Laboratory Manual*. If the isolated polynucleotide is an mRNA, it may be reverse transcribed into cDNA using conventional techniques, as described in Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*., cited *supra*.

[00111] An analyte may be a protein, polypeptide, lipid, carbohydrate, sugar, small molecule, or any other suitable molecule that can be detected with the methods and compositions provided herein. An analyte may be an enzyme or other protein. An analyte may be a drug or metabolite (e.g., anti-cancer drug, chemotherapeutic drug, anti-viral drug, antibiotic drug, or biologic). An analyte may be any molecule, such as a co-factor, receptor, receptor ligand, hormone, cytokine, blood factor, antigen, steroid, or antibody.

[00112] An analyte may be any molecule from any pathogen, such as a virus, bacteria, parasite, fungus, or prion (e.g., PrP^{Sc}). Examples of viruses include those from the families Adenoviridae, Flaviviridae, Hepadnaviridae, Herpesviridae, Orthomyxoviridae, Papovaviridae, Paramyxoviridae, Picornaviridae, Polyomavirus, Retroviridae, Rhabdoviridae, and Togaviridae. Specific examples of viruses include adenovirus, astrovirus, bocavirus, BK virus, coxsackievirus, cytomegalovirus, dengue virus, Ebola virus, enterovirus, Epstein-Barr virus, feline leukemia virus, hepatitis virus, hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis D virus, hepatitis E virus, herpes simplex virus (HSV), HSV type 1, HSV type 2, human immunodeficiency virus (HIV), HIV type 1, HIV type 2, human papilloma virus (HPV), HPV type 1, HPV type 2, HPV type 3, HPV type 4, HPV type 6, HPV type 10, HPV type 11, HPV

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type 16, HPV type 18, HPV type 26, HPV type 27, HPV type 28, HPV type 29, HPV type 30, HPV type 31, HPV type 33, HPV type 34, HPV type 35, HPV type 39, HPV type 40, HPV type 41, HPV type 42, HPV type 43, HPV type 44, HPV type 45, HPV type 49, HPV type 51, HPV type 52, HPV type 54, HPV type 55, HPV type 56, HPV type 57, HPV type 58, HPV type 59, HPV type 68, HPV type 69, influenza virus, JC virus, Marburg virus, measles virus, mumps virus, Norwalk virus, parovirus, polio virus, rabies virus, respiratory syncytial virus, retrovirus, rhinovirus, rotavirus, Rubella virus, smallpox virus, vaccinia virus, West Nile virus, and yellow fever virus.

[00113] Examples of bacteria include those from the genuses *Bordetella, Borrelia, Brucella, Campylobacter*, *Chlamydia, Clostridium, Corynebacterium, Enterococcus, Escherichia, Francisella, Haemophilus, Helicobacter, Legionella, Leptospira, Listeria, Mycobacterium*, *Mycoplasma, Neisseria, Pseudomonas*, *Rickettsia, Salmonella*, *Shigella*, *Staphylococcus, Streptococcus*, *Treponema, Vibrio, and Yersinia.* Specific examples of bacteria include *Bordetella par apertussis, Bordetella pertussis, Borrelia burgdorferi, Brucella abortus, Brucella canis, Brucella melitensis, Brucella suis, Campylobacter jejuni, Chlamydia pneumoniae, Chlamydia psittaci, Chlamydia trachomatix, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium tetani, Corynebacterium diphtheriae, Enterococcus faecalis, Enterococcus faecium, Escherichia coli*, *Francisella tularensis, Haemophilus influenzae, Helicobacter pylori, Legionella pneumophila, Leptospira interrogans, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Mycobacterium ulcerans, Mycoplasma pneumoniae, Neisseria gonorrhoeae, Neisseria meningitidis, Pseudomonas aeruginosa, Rickettsia rickettsii, Salmonella choleraesuis, Salmonella dublin, Salmonella enteritidis, Salmonella typhi, Salmonella typhimurium, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus agalactiae, Streptococcus pneumoniae, Streptococcus pyogenes, Treponema pallidum, Vibrio cholerae, Yersinia pestis, and Yersinia enterocolitica.*

[00114] Examples of parasites include those from the genuses *Acanthamoeba, Babesia, Balamuthia, Balantidium, Blasocystis, Cryptosporidium, Dientamoeba, Entamoeba, Giardia, Isospora, Leishmania, Naegleria, Pediculus, Plasmodium, Rhinosporidium, Sarcocystis,*

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Schistosoma, Toxoplasma, Trichomonas, and *Trypanosoma.* Specific examples of parasites include *Babesia divergens, Babesia bigemina, Babesia equi, Babesia microfti, Babesia duncani, Balamuthia mandrillaris, Balantidium coli, Dientamoeba fragilis, Entamoeba histolytica, Giardia lamblia, Isospora belli, Naegleria fowleri, Pediculus humanus, Plasmodium falciparum, Plasmodium knowlesi, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, Rhinosporidium seeberi, Sarcocystis bovihominis, Sarcocystis suihominis, Schistosoma mansoni, Toxoplasma gondii, Trichomonas vaginalis, Trypanosoma brucei,* and *Trypansoma cruzi.*

[00115] Examples of fungi include those from the genuses *Apophysomyces, Aspergillus, Blastomyces, Candida, Cladosporium, Coddidioides, Cryptococcos, Exserohilum, Fusarium, Histoplasma, Pichia, Pneumocystis, Saccharomyces, Sporothrix, Stachybotrys,* and *Trichophyton.* Specific examples of fungi include *Aspergillus fumigatus, Aspergillus flavus, Aspergillus clavatus, Blastomyces dermatitidis, Candida albicans, Coccidioides immitis, Crytptococcus neoformans, Exserohilum rostratum, Fusarium verticillioides, Histoplasma capsulatum, Pneumocystis jirovecii, Sporothrix schenckii, Stachybotrys chartarum,* and *Trichophyton mentagrophytes.*

[00116] In some cases, the methods provided in this disclosure may be used to detect any one of the analytes described above, or elsewhere in the specification. In some cases the methods provided in this disclosure may be used to detect panels of the analytes described above, or elsewhere in the specification. For example, a panel may comprise an analyte selected from the group consisting of any 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100 or more analytes described above or elsewhere in the specification.

[00117] An analyte may be obtained from any suitable location, including from organisms, whole cells, cell preparations and cell-free compositions from any organism, tissue, cell, or environment. Analytes may be obtained from environmental samples, biopsies, aspirates, formalin fixed embedded tissues, air, agricultural samples, soil samples, petroleum samples, water samples, or dust samples. In some instances, an analyte may be obtained from bodily fluids which may include blood, urine, feces, serum, lymph, saliva, mucosal secretions, perspiration, central nervous system fluid, vaginal fluid, or semen. Analytes may also be

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obtained from manufactured products, such as cosmetics, foods, personal care products, and the like. Analytes may be the products of experimental manipulation including, recombinant cloning, polynucleotide amplification, polymerase chain reaction (PCR) amplification, purification methods (such as purification of genomic DNA or RNA), and synthesis reactions.

[00118] More than one type of analyte may be detected in each multiplexed assay. For example, a polynucleotide, a protein, a polypeptide, a lipid, a carbohydrate, a sugar, a small molecule, or any other suitable molecule may be detected simultaneously in the same multiplexed assay with the use of suitable reagents. Any combination of analytes may be detected at the same time.

[00119] Detection of an analyte may be useful for any suitable application, including research, clinical, diagnostic, prognostic, forensic, and monitoring applications. Exemplary applications include detection of hereditary diseases, identification of genetic fingerprints, diagnosis of infectious diseases, cloning of genes, paternity testing, criminal identification, phylogeny, anti-bioterrorism, environmental surveillance, and DNA computing. For example, an analyte may be indicative of a disease or condition. An analyte may be used to make a treatment decision, or to assess the state of a disease. The presence of an analyte may indicate an infection with a particular pathogen, or any other disease, such as cancer, autoimmune disease, cardiorespiratory disease, liver disease, digestive disease, and so on. The methods provided herein may thus be used to make a diagnosis and to make a clinical decision based on that diagnosis. For example, a result that indicates the presence of a bacterial polynucleotide in a sample taken from a subject may lead to the treatment of the subject with an antibiotic.

[00120] In some cases the methods and compositions of the invention may be used to detect at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes. In some cases the methods and compositions of the invention may be used to detect 7-50, 8-40, 9-30, 10-20, 10-15, 8-12, or 7-12 analytes.

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[00121] In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis.

IV. Signals

[00122] The methods presented in this disclosure may be used with any quantifiable signal. In some cases, this disclosure provides coding schemes and methods to encode an infinite number of targets without degeneracy, using a single component of a signal (e.g., intensity). For example, as described above and in Example 3, a coding scheme may rely on a multiplicity of signal intensity without consideration of color. Although fluorescent probes have been used to illustrate this principle, the coding scheme is equally applicable to any other method providing a quantifiable signal, including an electrochemical signal and a chemiluminescent signal, as described elsewhere in this disclosure.

[00123] The methods presented in this disclosure may also utilize the measurement of a signal in at least two dimensions, also referred to as the measurement of at least two components of a signal. In comparison to the coding scheme described in the paragraph above, which relies on, for example, signal intensity to differentiate between analytes, utilization of at least two components of a signal (e.g., color and intensity) allows the generation of more unique codes per unit of signal intensity bandwidth. When at least two components of a signal are utilized, a cumulative measurement of the at least two components may be obtained for a single sample volume. For example in the coding scheme described in Table 5, the presence of each analyte results in a particular intensity (one component of the signal) in each of the four colors (a second component of a signal). The combination of these constituent signals leads to a cumulative signal that may be measured by measuring an intensity at each wavelength or range of wavelengths. The corresponding coding scheme or decoding matrix may then be used to convert the cumulative measurement into a determination of the presence or absence of an analyte.

[00124] In some cases, a quantifiable signal comprises a waveform that has both a frequency (wavelength) and an amplitude (intensity). A signal may be an electromagnetic signal. An electromagnetic signal may be a sound, a radio signal, a microwave signal, an infrared signal, a visible light signal, an ultraviolet light signal, an x-ray signal, or a gamma-ray signal. In some cases, an electromagnetic signal may be a fluorescent signal, for example a fluorescence emission spectrum that may be characterized in terms of wavelength and intensity.

[00125] In certain portions of this disclosure, the signal is described and exemplified in terms of a fluorescent signal. This is not meant to be limiting, and one of ordinary skill in the art will readily recognize that the principles applicable to the measurement of a fluorescent signal are also applicable to other signals. For example, like fluorescent signals, any of the electromagnetic signals described above may also be characterized in terms of a wavelength and an intensity. The wavelength of a fluorescent signal may also be described in terms of color. The color may be determined based on measuring intensity at a particular wavelength or range of wavelengths, for example by determining a distribution of fluorescent intensity at different wavelengths and/or by utilizing a band pass filter to determine the fluorescence intensity within a particular range of wavelengths. Such band pass filters are commonly employed in a variety of

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laboratory instrumentation, including quantitative PCR machines. Intensity may be measured with a photodetector. A range of wavelengths may be referred to as a "channel."

[00126] In some cases, the methods provided in this disclosure may be used with any signal where the cumulative signal scales with the constituent signals of the same color, frequency, absorption band, and so on. However, the cumulative signal need not be digital or scale linearly with the number and intensity of the constituent signals. For example, if the physical principle of measurement is absorption, the cumulative attenuation is a product of constituent attenuations while the constituent concentrations are additive, due to the exponential nature of the Beer-Lambert law. The logarithm of the cumulative attenuation will then scale linearly with constituent concentrations in each absorption band (the equivalent of color, if fluorescent detection is used). The methods of the invention are therefore applicable. The methods of the invention may also be used with chemiluminescent signals and electrochemical signals.

[00127] The number of signals, and the number of dimensions or components measured, may also be expanded beyond the numbers shown in the exemplary embodiments of the invention, leading to an expansion in multiplexing capability. The exemplary embodiments provided in this disclosure utilize coding schemes constructed utilizing a fluorescent signal with one or two components measured: wavelength and/or intensity. The number of analytes that can be encoded can be increased by increasing the number of wavelengths and/or intensities. The number of analytes that can be encoded can also be increased by increasing the number of signals, for example by combining a fluorescent signal with an electrochemical signal or a FRET signal (fluorescence resonance energy transfer).

[00128] In some cases, more than two components of a signal may be measured. For example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, 20 or more components of a signal may be measured. At least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, or 20 components of a signal may be measured. At least 2, but fewer than 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, or 20 components of a signal may be measured. In some cases, 2-3, 2-4, 2-5, 2-6, 3-5, 3-6, 3-8, or 5-10 components of a signal may be measured. These additional

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components may include kinetic components, such as a rate of signal decay and rate of photobleaching.

[00129] If a fluorescent signal is employed, the number of analytes that can be encoded may be further expanded by utilizing additional fluorophores. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores may be used. In some cases, fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores may be used. In some cases, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 fluorophores may be used.

[00130] Generally, the number of analytes that can be encoded may be further expanded by utilizing additional first values (e.g., intensities) that are values or ranges of values from a first component of a signal. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more first values that are values or ranges of values from a first component of a signal may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more first values that are values or ranges of values from a first component of a signal may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 first values that are values or ranges of values from a first component of a signal may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 first values that are values or ranges of values from a first component of a signal may be used.

[00131] In instances in which intensity is a component of a signal that is quantified, such as when a fluorescent signal is utilized, the presence of an analyte may be encoded using a variety of intensities or ranges of intensities. For example, a coding scheme may utilize 1, 2, 3,

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4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more intensities or ranges of intensities. A coding scheme may utilize at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more intensities or ranges of intensities. A coding scheme may utilize fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 intensities or ranges of intensities. In some cases, a coding scheme may utilize 2-20, 2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9, 2-10, 3-4, 3-5, 3-6, 3-7, 3-8, 3-9, 3-10, 4-5, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, or 5-10 intensities or ranges of intensities.

[00132] The number of analytes that can be encoded may be further expanded by utilizing additional second values (e.g., wavelengths) that are values or ranges of values from a second component of a signal. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second values that are values or ranges of values from a second component of a signal may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more second values that are values or ranges of values from a second component of a signal may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 second values that are values or ranges of values from a second component of a signal may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 second values that are values or ranges of values from a second component of a signal may be used.

[00133] In instances in which wavelength is a component of a signal that is quantified, such as when a fluorescent signal is utilized, the presence of an analyte may be encoded using a variety of wavelengths or ranges of wavelengths. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more wavelengths or ranges of wavelengths may

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be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more wavelengths or ranges of wavelengths may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 wavelengths or ranges of wavelengths may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 wavelengths or ranges of wavelengths may be used.

[00134] In some cases, when degeneracy is eliminated, the methods of the invention are capable of detecting the presence or absence of at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more analytes in a single volume when each reagent used to generate a signal in the volume generates only one second value (e.g., each reagent, emits light at only one wavelength).

[00135] In other cases, when degeneracy is eliminated, the methods of the invention are capable of detecting the presence or absence of at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more analytes in a single volume using a total of four second values in that volume (e.g., a total of four wavelengths or ranges or wavelengths, which might be implemented by using four spectrally resolvable fluorophores).

[00136] As described throughout the specification, the assay provided herein utilizes cumulative measurements on a sample. A cumulative measurement may be, for example, a single measurement of intensity values, or a measurement of intensity values at one or more wavelengths or ranges of wavelengths. A plurality of cumulative measurements may be obtained. For example, an intensity may be measured at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more wavelengths or ranges of wavelengths. An intensity may be measured at at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more wavelengths or ranges of wavelengths. An intensity may be

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measured at less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 wavelengths or ranges of wavelengths.

[00137] More generally a cumulative measurement may be obtained for any quantifiable component of a signal, and for any quantifiable component of a signal at another quantifiable component of a signal. For example, at least a first component of a signal may be measured at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second components of a signal. At lest a first component of a signal may be measured at at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second components of a signal. At least a first component of a signal may be measured at less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 second components of a signal.

[00138] As is apparent from this disclosure, each analyte to be detected can be encoded as a code utilizing any number of suitable components of a signal or any number of signals. For example, each analyte to be detected can be encoded in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more components of a signal or signals. In some cases, each analyte to be detected can be encoded in at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more components of a signal or signals. In some cases, each analyte to be detected can be encoded in fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 components of a signal or signals. In some cases, 1-4, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 components of signal or signals may be used to encode each analyte. Each analyte in a coding scheme may be encoded

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by the same number of components of a signal, or signals, or different numbers of components of a signal, or signals.

A. Probes, Primers, Fluorophores, and Quenchers

[00139] Some of the methods provided in this disclosure utilize a reagent that generates a signal in the presence of an analyte. Any suitable reagent may be used with the invention. Generally, a reagent will have an analyte-specific component and a component that generates a signal in the presence of the analyte. In some cases, these reagents are referred to as probes. The probes may be hybridization probes. The hybridization probes may be an oligonucleotide probe attached to a fluorophore and a quencher (e.g., a TAQMAN probe).

[00140] The methods of the invention may use one or more reagents or probes to detect the presence or absence of each analyte. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more or probes may be used to detect the presence or absence of each analyte. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 probes may be used to detect the presence or absence of each analyte. In some cases, fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, , 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 probes may be used to detect the presence or absence of each analyte. In some cases, the number of probes used to detect the presence or absence of each analyte is 1-10, 2-9, 2-8, 2-7, 2-6, 2-5, 2-4, 1-3, or 1-4.

[00141] In some cases, a sample is contacted with 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more probes to detect the presence or absence of all analytes. In some cases, a sample is contacted with at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more probes to detect the presence or absence of all analytes. In some cases, a sample is contacted with fewer than 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42,

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43, 44, 45, 46, 47, 48, 49, or 50 probes to detect the presence or absence of all analytes. In some cases, the number of probes that a sample is contacted with to detect the presence or absence of all analytes is 4-50, 4-40, 4-30, 4-20, 5-15, 5-10, or 3-10.

[00142] As described above, oligonucleotide probes attached to a fluorophore and a quencher may be used to detect the presence of an analyte in a polynucleotide amplification assay. So long as the quencher and the fluorophore are in proximity, the quencher quenches the fluorescence emitted by the fluorophore upon excitation by a light source. The sequence of the oligonucleotide probe is designed to be complementary to a polynucleotide sequence present in an analyte, and therefore capable of hybridizing to the polynucleotide sequence present in the analyte. Hybridization of the oligonucleotide probe may be performed in a nucleic acid amplification reaction comprising primers (e.g., a polymerase chain reaction). Upon extension of the primers by a DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and the quencher into the medium. The proximity between the fluorophore and the quencher is broken and the signal from the fluorophore is no longer quenched. Thus, the amount of fluorescence detected is a function of the amount of analyte present. If no analyte is present, the probe will not hybridize to an analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be produced.

[00143] Oligonucleotide probes may have one or a plurality of fluorophores and quenchers per probe. For example, in some embodiments an oligonucleotide probe may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores. An oligonucleotide probe may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores. An oligonucleotide probe may comprise fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores.

[00144] An oligonucleotide probe may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40,

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41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more quenchers. An oligonucleotide probe may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 quenchers. An oligonucleotide probe may comprise fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 quenchers.

[00145] Attachment of probes and quenchers to a probe may be performed in the same reaction or in serial reactions. A series of reactions may be performed to label probes with at least one fluorophore and the reaction products may be mixed to generate a mixture of probes with different fluorophores.

[00146] If an oligonucleotide probe comprises two or more fluorophores, these fluorophores may be arranged to allow Förster (Fluorescence) resonance energy transfer (FRET) to occur between the fluorophores. Briefly, FRET is a mechanism of energy transfer between fluorophores. Using FRET-based probes allows one excitation source to generate two different fluorescence emission signals by excitation of a single fluorophore. For example, the methods described herein may used paired probes, where one probe in the pair is attached to a fluorophore and a quencher and a second probe is attached to two fluorophores (in close enough proximity for FRET to occur) and a quencher. Any combination of fluorophores and quenchers that provide for FRET may be used. Such an approach doubles the number of fluorescent probes that may be used with an excitation source, because a single excitation source can be used to produce two signals: one from the probe with one fluorophore and one from the probe with two fluorophores in close enough proximity to provide for FRET. For example, using the encoding method described in Table 8, the number of unambiguously detectable analytes could be increased from 16 to 32 by pairing each of the probes in Table 8 with a corresponding FRET probe.

[00147] The primers may be specific for a particular analyte and capable of amplifying a region complementary to a probe. In some cases, the number of pairs of primers used is equivalent to the number of probes. In other cases, the number of probes used may exceed the number of primer pairs used. In still other cases, the number of primer pairs used may exceed

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the number of probes used. In some cases a sample to be analyzed is contacted with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more pairs of primers. In some cases a sample to be analyzed is contacted with at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more pairs of primers. In some cases a sample to be analyzed is contacted with fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 pairs of primers. In some cases, the number of pairs of primers is 2-10, 3-15, 4-20, 3-10, 4-10, 5-10, 6-8, or 6-10.

[00148] The primers may amplify regions of a polynucleotide in which different numbers of hybridization probes hybridize. For example, at least one pair of primers may amplify a region complementary to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 hybridization probes. In some cases, all of said pairs of primers may amplify a region complementary to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 hybridization probes.

[00149] In one example, a first probe may be labeled with the fluorophore 5'-fluorescein amidite (5'-FAM) and the quencher black hole quencher 1 (BHQ-1). A second probe may be labeled with 5'-FAM in close proximity (e.g., attached to) to cyanine 5.5 (Cy5.5) and the quencher black hole quencher 3 (BHQ-3). Upon digestion of the probe, via the nuclease activity of the polymerase, two fluorescent signals are generated from a single excitation wavelength (e.g., 470 nm). The fluorophore from the first probe will fluoresce at about 520 nm. The fluorophores on the second probe undergo FRET. The donor fluorophore, FAM is excited by the excitation light source (e.g., 470 nm) and transfers its energy to the acceptor fluorophore, Cy5.5. The acceptor fluorophore emits at about 705 nm. These fluorophores and quenchers are merely exemplary. Any fluorophores that can undergo FRET and any quenchers that can quench

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fluorescence are suitable for use with the invention. Methods for producing FRET-based probes are described in Jothikumar et al., BioTechniques, 2009, 46(7):519-524.

[00150] In some examples, a single hybridization probe may be used for each analyte. In order to utilize multi-color encoding (e.g., as shown in Table 3), each probe may be labeled with a plurality of fluorophores at pre-determined ratios. The ratio may be determined so that a positive control signal provides the same intensity as other positive control signals of the same intensity, in the same color. This approach reduces the number of probes that must be synthesized and may be less expensive to deploy than methods utilizing multiple probes. Moreover, in the case of hybridization probes, it is easier to fit one hybridization probe to an analyte sequence than multiple probes.

[00151] Although many aspects of the invention are exemplified using nucleic acid-based probes, one of ordinary skill in the art will readily recognize that other forms of probes would work equally well with the invention described in this disclosure. For example, a binding molecule specific to an analyte could be used as a probe. Non-limiting exemplary binding molecules include an antibody recognizing an analyte, and generating a signal in the presence of an analyte.

[00152] In embodiments of the invention that utilize fluorescent labels, any suitable fluorescent label may be used. Exemplary fluorescent labels suitable for use with the invention include rhodamine, rhodol, fluorescein, thiofluorescein, aminofluorescein, carboxyfluorescein, chlorofluorescein, methylfluorescein, sulfofluorescein, aminorhodol, carboxyrhodol, chlororhodol, methylrhodol, sulforhodol; aminorhodamine, carboxyrhodamine, chlororhodamine, methylrhodamine, sulforhodamine, and thiorhodamine, cyanine, indocarbocyanine, oxacarbocyanine, thiacarbocyanine, merocyanine, cyanine 2, cyanine 3, cyanine 3.5, cyanine 5, cyanine 5.5, cyanine 7, oxadiazole derivatives, pyridyloxazole, nitrobenzoxadiazole, benzoxadiazole, pyrene derivatives, cascade blue, oxazine derivatives, Nile red, Nile blue, cresyl violet, oxazine 170, acridine derivatives, proflavin, acridine orange, acridine yellow, arylmethine derivatives, auramine, crystal violet, malachite green, tetrapyrrole derivatives, porphin, phtalocyanine, and bilirubin. Exemplary quenchers include black hole

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quenchers, such as BHQ-0, BHQ-1, BHQ-2, BHQ-3; ATTO quenchers, such as ATTO 540Q, ATTO580Q, and ATTO612Q; and the like.

[00153] The fluorophores that may be used with the invention are not limited to any of the fluorophores described herein. For example, fluorophores with improved properties are continually developed, and these fluorophores could readily be used with the methods provided in this disclosure. Such improved fluorophores include quantum dots, which may emit energy at different wavelengths after being excited at a single wavelength. The advantage of using such fluorophores is that only a single excitation source is needed, but many different signals may be quantified, for example in terms of color and intensity. Moreover, fluorophores with narrow emission spectra would be particularly useful with the method described herein, as such fluorophores could be included in multiplex assays with minimal or no overlap between their emission spectra, thereby offering many more "colors" and boosting significantly the overall number of coded analytes.

[00154] In some cases, one or more reagents are lyophilized. Any suitable reagent may be lyophilized. For example, probes, primers, enzymes, antibodies, or any other reagent used for detection may be lyophilized. In some cases, a sample comprising an analyte may also be lyophilized. Lyophilization may be useful, for example, when distributing reagents and/or samples in developing regions where access to cold storage is expensive, not readily available, or not reliably available. In one example, lyophilized reagents comprise any probe or analyte-specific reagent described in this disclosure or otherwise suitable for use with the invention. In another example, lyophilized reagents comprise PCR primers. In yet another example, lyophilized reagents comprise reagents suitable for performing a PCR reaction.

V. Analytical Techniques and Instrumentation

[00155] The methods provided herein are suitable for use with a variety of detection methods. For example, the methods may be applied using an analytical technique that measures the wavelength and intensity of a fluorescent signal. This may be accomplished by measuring the intensity of a signal across a spectrum of wavelengths, or by using band pass filters that restrict the passage of certain wavelengths of light, thereby allowing only light of certain wavelengths to reach a photodetector. Many real-time PCR and quantitative PCR instruments

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comprise an excitation light source and band pass filters that enable the detection of fluorescent signals in four colors (e.g., blue, green, yellow, and red). Therefore, the methods of the invention can be readily applied using instruments widely used in the art. Importantly, the methods and compositions provided herein may be used to detect multiple analytes by obtaining a cumulative measurement on a single solution. No separation is necessary. The invention does not require the use of beads or a solid phase. Of course, one of ordinary skill in the art would understand that the invention could be used with separation, beads, or a solid phase, if desired.

[00156] **Fig. 5** shows two examples of instrumental configurations that may be used to obtain cumulative measurements useful for the methods of the invention. With reference to **Fig. 5**, an instrument with a detector configured to detect both wavelength and intensity (e.g., a fluorometer) is shown in **501.** With reference to **501**, at least one excitation light source **502** is directed into a chamber **503** containing analytes and reagents that generate a signal in the presence of the analytes **504.** As described elsewhere, the analyte may be a nucleic acid and the reagents generating the signal may be hybridization probes comprising a fluorophore and a quencher. If the analyte is present, an emission signal **505** is generated. In the configuration shown in **501**, the wavelength and intensity of this emission signal are measured across a spectrum by a detector capable of generating a fluorescence emission spectrum **506.**

[00157] The wavelength and intensity may also be determined using a combination of a photodetector and band pass filters. This configuration is used in several thermal cyclers known in the art. With reference to **Fig. 5**, an instrument with band pass filters and a photodetector is depicted in **507.** With reference to **507**, at least one excitation light source **508** is directed into a chamber **509** containing analytes and reagents that generate a signal in the presence of the analytes **510.** As described elsewhere, the analyte may be a nucleic acid and the reagents generating the signal may be hybridization probes comprising a fluorophore and a quencher. If the analyte is present, an emission signal **511** is generated. In the configuration shown in **507**, band pass filters **512, 513,** and **514** are used to restrict the passage of light to light within certain ranges of wavelengths. For example band pass filter **512** may restrict the passage of light to light within wavelength range 1. Band pass filter **513** may restrict the passage of light to light within wavelength range 2. Band pass filter **514** may restrict the passage of light to light within

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wavelength range 3. Any number of band pass filters (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more) may be used with the invention. In addition, the positions of the band pass filters in **507** are not meant to be limiting. In some cases, emitted light may be passed through more than one band pass filter at any time. In other cases, the band pass filters may be configured so that all emitted light passes through only one band pass filter at a time. After passage through a band pass filter, filtered light **515, 516,** and **517** is detected by a photodetector. For example, filtered light **515** may be light within wavelength range 1, filtered light **516** may be light within wavelength range 2, and filtered light **517** may be light within wavelength range 3. The intensity of each of the filtered lights **515, 516,** and **517** may then be quantified by a photodetector **518.**

[00158] The methods described in this disclosure are compatible with a variety of amplification methods, including quantitative PCR (qPCR) methods, end point PCR methods, reverse transcriptase PCR, and digital PCR methods. Digital PCR methods (e.g., DROPLET DIGITAL (BIORAD) and DYNAMIC ARRAY (FLUIDIGM)) produce highly sensitive quantification of polynucleotide copy numbers. The methods provided herein can be easily integrated into these systems to significantly expand their throughput by allowing multiplexing in a droplet or a dynamic array.

[00159] Similarly, the methods described in this disclosure may be applied in a real-time PCR assay. For example, real-time data may be fully recorded as the PCR runs to completion. The end-point values may then be decoded to indicate the presence or absence of analytes. The individual cycle thresholds (Ct) values may be analyzed for each color by detecting the maxima of the second derivative of the real-time curve in that color. As a particular analyte is amplified, fluorophores are released from its corresponding probes in the same ratios as the color multiplicities in the coding of that sequence. Hence, e.g. a code of 1100 will have the same Ct in blue and green, while a code of 1400 will have its green Ct precede its blue Ct by 2 cycles. This additional data enables determination of the identity and starting quantity of each analyte.

[00160] The methods described herein may also be used directly on a tissue sample. For example, a tissue sample may be obtained and photolithographic methods may be used to build wells directly onto the tissue sample. The tissue sample may be fixed prior to building the wells.

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The tissue sample may then be analyzed by dispensing appropriate reagents into the wells in the tissue sample. The encoding and decoding methods described in this disclosure may be used for the multiplexed detection of analytes within the wells etched into the tissue. Each well may correspond to a single cell or a few cells, providing excellent spatial resolution when analyzing different portions of a tissue for an analyte. This method may be used to detect analytes in different areas of the same tissue.

[00161] In some cases, instruments may be modified or constructed, for example, to provide additional excitation light sources, at multiple wavelength, and/or to provide additional band pass filters or a capability of determining a complete spectrum. Including additional excitation sources would allow for the excitation of a larger variety of fluorophores. Including additional band pass filters, or modifying or constructing an instrument capable of determining an entire spectrum allows detection of a wider variety of emissions from fluorophores. These techniques can be used to increase the number of fluorophores that can be used with the methods described in this disclosure and, accordingly, to increase the number of analytes that be simultaneously detected.

[00162] In some cases, the methods described in this disclosure utilize end-point PCR methods. However, in some cases a result may be obtained before the end-point. This may be advantageous when, for example, results are needed as soon as possible. For example, in one case calibration experiments are performed in which different starting concentrations of an analyte are analyzed to determine the cycle number at which the signal becomes saturated, as a function of the starting amount of the analyte. Using this data, a computer monitoring a PCR reaction in real-time can be programmed to search for saturation up to the maximal cycle number according to the limit of detection (LOD) of the particular system. If there is no amplification of an analyte (e.g., other than a positive control, if present), by the maximal cycle number, the result for that analyte is negative. If there is amplification of the analyte by the maximal cycle number, the result for that analyte is positive and the saturation intensity in each color may be used to decode the result using the coding scheme. In both cases, there would be no need to run the PCR reaction beyond the number of cycles set by the LOD for that instrument. Although PCR has been used to illustrate this quantitative method, one of ordinary skill in the art will readily

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recognize that similar principles could be applied in any catalytic system where the starting amount of an analyte limits the rate of reaction, and the rate of reaction can be measured and reported by an evolution in a signal intensity over time.

VI. Compositions and Kits

[00163] This disclosure also provides compositions and kits for use with the methods described herein. The compositions may comprise any component, reaction mixture and/or intermediate described herein, as well as any combination. For example, the disclosure provides detection reagents for use with the methods provided herein. Any suitable detection reagents may be provided, including hybridization probes labeled with a fluorophore and a quencher and primers, as described elsewhere in the specification.

[00164] In some cases, compositions comprise reagents for the detection of at least seven analytes using four fluorophores. In some cases, compositions comprise reagents for the detection of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using four fluorophores. In some cases, compositions comprise reagents for the detection of at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using five fluorophores. In some cases, compositions comprise reagents for the detection of at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using six fluorophores. In some cases, compositions comprise reagents for the detection of at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using seven fluorophores.

[00165] In some cases the compositions comprise primers. The compositions may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 pairs of primers.

[00166] The invention also provides kits for carrying out the methods of the invention. Accordingly, a variety of kits are provided in suitable packaging. The kits may be used for any

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one or more of the uses described herein, and, accordingly, may contain instructions for detecting the presence or absence of each analyte of a plurality of analytes. A kit may comprise a coding scheme, or a decoding matrix, to assist the user in converting a cumulative measurement to a result indicating the presence or absence of each of a plurality of analytes. A kit may be a diagnostic kit, for example, a diagnostic kit suitable for the detection of any analyte, including the analytes recited herein. A kit may contain any of the compositions provided in this disclosure, including those recited above.

[00167] **Fig. 6** shows schematics of exemplary kits of the invention. With reference to **Fig. 6**, a kit comprising a kit body **601** is shown that contains reagents for the detection of analytes (e.g., polynucleotide probes comprising a fluorophore and a quencher) **602** and primers for the amplification of a nucleic acid **603.** The kit may also contain any other reagents, such as reagents suitable for performing an amplification reaction **604.** In the embodiment depicted in **601**, the reagents for the detection of analytes and the primers are each contained in a single volume (e.g., tube or a bottle). However, these reagents may also be provided in separate volumes, as depicted the kit body shown in **605.** The kit body **605** contains seven separate volumes. Three volumes (**606, 607,** and **608**) contain reagents for the detection of analytes (or mixtures of such reagents) and three volumes (**609, 610,** and **611**) contain primers (or mixtures of primers). The kit may also contain reagents suitable for performing an amplification reaction **604.**

[00168] **Fig. 6** is provided for illustrative purposes only. Any number of reagents for the detection of analytes (e.g., probes) and any number of primers may be included in a single tube or bottle, as appropriate for the application. For example, in some embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more reagents for the detection of analytes may be included in a single bottle. In other cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more primers for amplification may be included in a single bottle or tube. In some cases, primers and probes may be provided in the same bottle or tube. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23,

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24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more probes may be provided per pair of primers. In other cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more primers may be provided per pair of probes. The kits may contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more bottles or tubes. Control analytes, and their associated detection reagents and primers, may also be included in the kit. These may be included in a separate bottle or tube or, for example, included within the bottle or tube providing reagents for an amplification reaction **604**. As described elsewhere in the specification, any reagent in the kits may be lyophilized.

VII. Systems and Software

[00169] The methods of this disclosure may also be performed as part of a system. For example, one or more steps of the methods described herein may be performed by software contained on a computer readable medium. The software may be used to encode analytes, check for degeneracy, reduce or eliminate degeneracy, and/or produce a decoding matrix. The software may also be used to convert cumulative measurements made on an instrument (e.g., a PCR machine) into information concerning the presence or absence of an analyte. For example, with reference to **Fig. 4**, a computer **401** may be connected to an input device **403**, a display **402**, and a printer **404**. The computer may execute one or more of the steps of any of the methods described in this disclosure, including, for example, encoding methods **410**, analysis methods **420**, and decoding methods **430.** A computer may reduce or eliminate degeneracy, for example as part of the encoding methods **410.** Similarly, a computer may generate a coding schme that is non-degenerate by design using, for example, mathematical iteration. A computer may also be connected to a network **405**. The network may be a local network and/or the Internet. The network may be used to perform the methods of the invention on multiple computers in different locations. For example, one computer may perform the encoding, another computer may perform the measuring, and yet another computer may perform the decoding. A computer may perform any or all of these methods. The network may be used to transmit information obtained using the methods described in this disclosure throughout the world, for example across a border.

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[00170] In one example, the software is embedded in the instrument making the measurement, such as a thermal cycler. In another example, the software is installed on a computer attached to the instrument making the measurement, such as a computer attached to a thermal cycler. In another example, the software is in the "cloud" -- i.e., on a computer that another computer may communicate with through a network.

[00171] Each of the elements described above may be connected to a controller that communicates with each of the elements and coordinates their actions. For example, a controller may initiate the encoding of a series of analytes into values of color and intensity. As described throughout this disclosure, the controller can execute software to reduce or eliminate degeneracy by removing one or more potential analyte codes from the encoding matrix. The controller may then automatically order probes corresponding to this code from a manufacturer of probes. Alternatively, the controller may operate an oligonucleotide synthesis instrument that can synthesize the appropriate probes. After analysis of the sample on the instrument (which may also be automated), the controller may process the results of the analysis using the decoding matrix. The controller may then provide these results to a user.

VIII. Services

[00172] The methods provided herein may also be performed as a service. For example, a service provider may obtain the identity of a plurality of analytes that a customer wishes to analyze. The service provider may then encode each analyte to be detected by any of the methods described herein and provide appropriate reagents to the customer for the assay. The customer may perform the assay and provide the results to the service provider for decoding. The service provider may then provide the decoded results to the customer. The customer may also encode analytes, generate probes, and/or decode results by interacting with software installed locally (at the customer's location) or remotely (e.g., on a server reachable through a network). Exemplary customers include clinical laboratories, physicians, manufacturers of food and consumer products, industrial manufacturers (e.g., petroleum companies) and the like. A customer or party may be any suitable customer or party with a need or desire to use the methods, systems, compositions, and kits of the invention.

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EXAMPLES

Example 1: General Materials & Methods

1. General Reagents

[00173] TE Buffer, pH7 (LIFE TECHNOLOGIES, Carlsbad, CA); UltraPure RNAse-free Water (LIFE TECHNOLOGIES, Carlsbad, CA); Taq 5x Master Mix (FISHER SCIENTIFIC COMPANY, Tustin, CA).

2. DNA Sequences, Primers, and Probes

[00174] Five nucleic acids from organisms of clinical relevance were chosen for the exemplary study: (1) human immunodeficiency virus 1 (HIV-1); (2) *Plasmodium falciparum* (Malaria); (3) herpes simplex virus-2 (HSV-2); (4) *Mycobacterium tuberculosis* (TB); (5) and dengue virus type 3 (dengue fever).

[00175] Two regions of diagnostic relevance from the HIV-1 genome, p17 and poly-protease, were selected from the Los Alamos National Laboratory HIV-1 reference sequence. A diagnostic sequence from the *Plasmodium falciparum* ChR7 gene was obtained from the UCSC *Plasmodium falciparum* Genome Browser. A sequence for Herpes Simplex Virus-2 was synthesized from the sequence obtained from the European Molecular Biology Library. Similarly a diagnostic sequence for the rpoB gene in *Mycobacterium tuberculosis* was synthesized from a sequence obtained from the European Molecular Biology Library. A PCR diagnostic sequence for Dengue Virus Type 3 was obtained from the National Institute of Health genetic sequence database.

[00176] Oligonucleotides were synthesized by INTEGRATED DNA TECHNOLOGIES (IDT). Probes and primer pairs were chosen for each analyte using OLIGOANALYZER, from IDT. Sense probes containing a fluorophore at the 5' end and a quencher at the 3' end were synthesized for all analytes. All oligonucleotides were lyophilized and reconstituted in TE buffer before use. Tables 9 - 14 show sequence information, target sequence, primers, and probes for each of the six analytes.

Table 9. HIV-1 polyprotease sequence information.

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Sequence Information	HIV-1 Poly protease 198mer synthesized from bases 2253 – 2550
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (3' to 5')	GGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGA GTTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTT ATCAAAGTAAGACAGTATGATCAGATACTCATAGAAATCTGTGGACATAA AGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGG
Forward Primer (3' to 5')	GGAAGCTCTATTAGATACAGGAGCAG
Reverse Primer (3' to 5')	CCAATTATGTTGACAGGTGTAGGTCC
Probe 1 (3' to 5')	**/56-FAM/**TGAGTTTGCCAGGAAGATGGAAACCA/3BHQ_1/

Table 10. HIV p17 sequence information.

Sequence Name	HIV-1 P17 199mer synthesized from bases 790 – 1186
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (3' to 5')	CAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATA TAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAG ACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAG AAAAAAGCACAGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGTCA
Forward Primer (3' to 5')	CAGCTACAACCATCCCTTCAGACA

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Reverse Primer (3' to 5')	TGACCTGATTGCTGTGTCCTGTGT
Probe 1 (3' to 5')	**/56-FAM/**AGCAACCCTCTATTGTGTGCATCAAAGG/3BHQ_1
Probe 2 (3' to 5')	**/5Cy3/**AAAGCACAGCAAGCAGCAGCTGA/3BHQ_2/

Table 11. *Plasmodium* ChR7 sequence information.

Sequence Name	*Plasmodium* ChR7 199mer synthesized from bases 1139138 - 1141223
Source	UCSC Plasmodium falciparum Genome Browser Gateway
Target sequence (3' to 5')	GCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAG AGGGAGCCTGAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTA AATTACCCAATTCTAAAGAAGAGAGGTAGTGACAAGAAATAACAATGCAA GGCCAATTTAAAACCTTCCCAGAGTAACAATTGGAGGGCAAGTCTGGTG
Forward Primer (3' to 5')	GCCTAACATGGCTATGACGGGTAA
Reverse Primer (3' to 5')	CACCAGACTTGCCCTCCAATTGTT
Probe 1 (3' to 5')	**/56-FAM/**ATTCCGGAGAGGGAGCCTGAGAAATA/3BHQ_1/
Probe 2 (3' to 5')	**/56-ROXN/**AAGGAAGGCAGCAGGCGCGTAAATTA/3BHQ_2/

Table 12. Herpes simplex virus 2 sequence information.

Sequence Name	HSV-2 193mer synthesized from HSV-2 genome

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Source	EMBL Bank AJ303204
Target sequence (3' to 5')	TCAGCCCATCCTCCTTCGGCAGTATGGAGGGTGTCGCGGCGGCGAGCCGC CGTCCCCAAAGACGTGCGGGTCGTACACGTACACGTACCAGGGCGGCGGG CCTCCGACCCGGTACGCTCTCGTAAATGCTTCCCTGCTGGTGCCGATCTG GGACCGCGCCGCGGAGACATTCGAGTACCAGATCGAACTCGG
Forward Primer (3' to 5')	TCAGCCCATCCTCCTTCGGCAGTAT
Reverse Primer (3' to 5')	CCGAGTTCGATCTGGTACTCGAATGT
Probe 1 (3' to 5')	**/56-FAM/**AAAGACGTGCGGGTCGTACACGTACA/3BHQ_1/
Probe 2 (3' to 5')	**/5Cy5/**TAAATGCTTCCCTGCTGGTGCCGAT/3IAbRQSp/

Table 13. *Mycobacterium tuberculosis* rpoB sequence information

Sequence Name	Mycobacterium Tuberculosis rpoB 200mer synthesized from Mycobacterium tuberculosis rpoB genome
Source	EMBL Bank GQ395623
Target sequence (3' to 5')	GAGTGCAAAGACAAGGACATGACGTACGCGGCCCCGCTGTTCGTCACGGC CGAGTTCATCAACAACAACACCGGCGAGATCAAGAGCCAGACGGTGTTCA TGGGTGACTTCCCGATGATGACCGAGAAGGGCACCTTCATCATCAACGGC ACCGAGCGCGTCGTGGTCAGCCAGCTGGTCCGCTCGCCCGGTGTGTACTT
Forward Primer (3' to 5')	GAGTGCAAAGACAAGGACATGACG

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Reverse Primer (3' to 5')	AAGTACACACCGGGCGAGC
Probe 1 (3' to 5')	**/56-FAM/**CGCTGTTCGTCACGGCCGAGTTCAT/3BHQ_1/
Probe 2 (3' to 5')	**/5Cy3/**AGATCAAGAGCCAGACGGTGTTCATG/3BHQ_2/
Probe 3 (3' to 5')	**/5Cy5/**AAGGGCACCTTCATCATCAACGGCA/3IAbRQSp/

Table 14. Dengue virus type 3 sequence information.

Sequence Name	Dengue Virus Type 3 200mer synthesized from Dengue Virus Type 3 genome
Source	GenBank M93130
Target sequence (3' to 5')	ATGCCAACTGTGATTGAGCACTTAGAAAGACTACAAAGGAAACATGGAGG AATGCTTGTGAGAAATCCACTCTCACGAAACTCCACGCACGAAATGTATT GGATATCCAATGGTACAGGCAACATCGTCTCATTCACAATGACACACAGG AGACCCACCATAGAGAAAGATGTGGATTTAGGAGCAGGAACCCGACATGT
Forward Primer (3' to 5')	ATGCCAACTGTGATTGAGCACT
Reverse Primer (3' to 5')	ACATGTCGGGTTCCTGCTCCTAAA
Probe 1 (3' to 5')	**/56-FAM/**ACAAAGGAAACATGGAGGAATGCTTGTGA/3BHQ_1/
Probe 2 (3' to 5')	**/5Cy3/**ACTCTCACGAAACTCCACGCACGAAA/3BHQ_2/
Probe 3 (3' to 5')	**/56-ROXN/**ACAATGACACACAGGAGACCCACCAT/3BHQ_2/

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Probe 4 (3' to 5')	**/5Cy5/**GGATATCCAATGGTACAGGCAACATCGT/3IAbRQSp/

3. Polymerase Chain Reactions

[00177] All PCR reactions were performed in a ROCHE 480 LIGHTCYCLER instrument. Forty-five thermal cycles were performed, with a 60 second hot-start at 95°C. The cycling conditions for denaturation, annealing, and extension were 45 seconds, 50 seconds, and 60 seconds at 95°C, 65°C, and 70°C, respectively. Each experiment was run in quintuplicate, with a reaction volume of 15 µL. Fluorescence measurements were obtained in the following channels after every annealing step: 483 nm to 533 nm (FAM), 523 nm to 568 nm (Cy3), 558 nm to 610 nm (ROX), and 615 nm to 670 nm (Cy5). Fluorescence measurements were also obtained after the hot-start and the end of the 45 thermal cycles. The change in the fluorescence intensity between these two measurements (after hot-start and at the end of the 45 cycles) determined the end-point fluorescent signal.

Example 2: Simultaneous Detection of Polynucleotide Markers from Pathogens Based on an Encoding Method Using More Than One Color Per Analyte

[00178] This example describes the simultaneous detection of up to four polynucleotides selected from HIV (2 polynucleotides), HSV-2, *Mycobacterium*, *Plasmodium*, and dengue virus. Fourteen PCR reactions were assembled, using the reagents described in Table 15.

Table 15. Reagents for 14 PCR reactions.

Reaction No.	Type of Reagent	Reagent	Concentration	Volume
1		UltraPure Water	-	34 µL
		Taq 5x Master Mix	-	42 µL
	Template	Poly Protease Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 nM	2 µL
		Protease RVS Primer	1 nM	2 µL
	Probe	Poly Protease Probe 1	1 nM	2 µL
2		UltraPure Water	-	28 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Malaria Template	15 nM	2 µL

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		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 nM	2 µL
		Protease RVS Primer	1 nM	2 µL
	Probe	Poly Protease Probe 1	1 nM	2 µL
3		UltraPure Water	-	32 µL
		Taq 5x Master Mix	-	42 µL
	Template	Malaria Template	15 nM	2 µL
	Primers	Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
	Probes	Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
4		UltraPure Water	-	26 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
	Probes	Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
5		UltraPure Water	-	32 µL
		Taq 5x Master Mix	-	42 µL
	Template	Herpes Template	17 nM	2 µL
	Primers	Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
	Probes	Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
6		UltraPure Water	-	26 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
	Probes	Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
7		UltraPure Water	-	30 µL
		Taq 5x Master Mix	-	42 µL

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	Template	TB Template	16 nM	2 µL
	Primers	TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
8		UltraPure Water	-	30 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
9		UltraPure Water	-	28 µL
		Taq 5x Master Mix	-	42 µL
	Template	Dengue Virus Template	17 nM	2 µL
	Primers	Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
10		UltraPure Water	-	22 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
11		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL

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		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease RVS Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
12		UltraPure Water	-	2 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
13		UltraPure Water	-	12 µL
		Taq 5x Master Mix	-	42 µL

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	Templates	Poly Protease Template	17 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
14		UltraPure Water	-	10 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
		Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
		Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL

[00179] Referring to Table 15, reactions 1, 3, 5, 7, and 9 (containing HIV polyprotease, *Plasmodium*, HSV, *Mycobacterium*, and dengue analytes, respectively) were positive controls that provided baseline fluorescence intensity for each polynucleotide analytes alone, in the absence of other analytes. The change in fluorescence intensity in each color was used to provide the expected cumulative intensity level for each color in the chromatogram **(Fig. 3)**, as described more fully below. Reactions 2, 4, 6, 8, and 10 were negative controls used to

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determine the extent of non-specific amplification. In these reactions, the primers provided were not specific for the analytes provided.

[00180] Experiments 11, 12, 13, and 14 were multiplex detection experiments. Encoding was performed as indicated in Table 16. The measured fluorescence intensity for each assay, in each color, was plotted as black circles in the chromatogram of **Fig. 3.**

Table 16. Coding scheme for detection of pathogens.

	FAM	Cy3	ROX	Cy5
HIV poly-protease	1	0	0	0
HIV p17	1	1	0	0
Plasmodium	1	0	1	0
HSV	1	0	0	1
Mycobacterium	1	1	0	1
Dengue Virus	1	1	1	1

[00181] To construct the chromatogram, the signals of the positive controls (reactions 1, 3, 5, 7, and 9) were used to assemble every possible combination of present sequences, in each color. The cumulative signals were plotted in a diagram, called a "chromatogram" (**Fig. 3**). Cumulative signals corresponding to all possible combinations of the same rank in the same color were organized into their own "band." Doing this for all four colors produced a level and band structure, against which the experimental results of each combination (reactions 11, 12, 13, and 14) could be evaluated. **Fig. 3** shows three replicates of a chromatogram constructed using the positive control samples, with experimental results 4112, 3121, and 3102 superimposed on the chromatogram. The experimental results are indicated by black dots.

[00182] The intensity value for each color in reactions 11-14 was measure in arbitrary fluorescence units (AFU), plotted on the chromatogram, and then assigned to a band based on two criteria. First, if an experimental result was within a band, then the result was assigned to the multiplicity of the band (e.g., the Cy3 value of 1 in the left-most chromatogram of **Fig. 3**). Second, if an experimental result was between two bands, a two-step analysis was employed. If the experimental result was between two legitimate results, the experimental result was assigned to the closest band. (e.g., the Cy5 value of 2 in the right-most chromatogram of **Fig. 3**). If the experimental result was between a legitimate result and an illegitimate result, the result was

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assigned to the band with the legitimate result (e.g., the FAM value of 3 in the middle chromatogram of **Fig. 3**). **Fig. 8** schematically shows a legitimate result of 4112 (top) indicating the presence of HIV polyprotease, *Plasmodium*, herpes simplex, and *Mycobacterium*; and an illegitimate result of 4110 (bottom), which cannot be decoded and indicates a malfunction in the assay.

[00183] Using these criteria, the output of Experiment 12 was designated 4112, which was the correct answer. The output of Experiment 14 could have been designated as 2121 or 3121, but 2121 is illegitimate, so the result must be 3121, which is the correct answer. The output of Experiment 13 could be designated as 3102 or 3101, both of which are legitimate, but the result is clearly closer to 2 than 1 in the Cy5 channel, so 3102, the correct answer, was designated as the result. These results provide experimental validation of the methods of the provided herein.

[00184] The results indicate a trend of slightly lower combined signal than what would be expected from a simple summation of positive-control signals. This may be because the combination of signals is not perfectly linear. This may be a result of additional effects for which the chromatogram construction does not account for at present. These effects may be related to the absolute concentration of quenchers. The higher the absolute concentration of quenchers, the larger the percentage of reaction volume they quench, so that the same percentage of released fluorophores fails to contribute as much as expected to the cumulative signal. One solution to this problem is to use low concentrations of probes, so that the percentile loss in signal is negligible, regardless of how much quencher is released.

[00185] **Fig. 3** shows that each rank containing multiple combinations has a relatively wide band. The width of the band is ultimately the difference between the highest and lowest cumulative signals for the positive control samples. If all positive controls in the particular color produced exactly the same fluorescence signal, then the width of each band would be zero. Since instead those signals are somewhat different, the resulting bands are relatively wide. However, there is a simple means to tighten the bands. Instead of loading all probes at the same concentration, as was done for the experiment above, the concentration of each probe can be adjusted so that each of the resulting positive-control signals is the same as the others in the same

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color. This approach would significantly tighten the bands, making it easier to determine the multiplicity of a signal for a sample containing analytes.

Example 3: Simultaneous Detection of Polynucleotide Markers from Pathogens Based on an Encoding Method Using One Color Per Analyte and Different Intensities

[00186] This example describes the simultaneous detection of up to three polynucleotides selected from dengue virus, HIV p17, and HIV polyprotease. The coding scheme was as depicted in Table 11. As shown in Table 17, each analyte was encoded as a single value of fluorescence intensity in a single color. In this case, as described elsewhere in this disclosure, the coding scheme is non-degenerate by design. This is achieved by assigning an intensity value to each analyte that is equal to the cumulative intensity values for the prior analytes, plus one. Of course, as described elsewhere in the specification, the initial assigned value can be greater than one, for example to better distinguish over noise. Similarly, the values assigned to each analyte can be greater than the cumulative intensity values for the prior analytes plus one, for example, to increase the separation between the intensities and enable more simple assignment of intensities.

Table 17. Coding scheme for detection of dengue virus, HIV polyprotease, and HIV p17.

	FAM
Dengue Virus	1
HIV p17	2
HIV polyprotease	4

[00187] The same reagents (e.g., templates, primers, and probes) used in Example 2 were used here. The fluorescence signals generated by the respective probes in positive-control end-point PCR reactions were measured and used to calculate the probe concentrations that would produce 1x, 2x, and 4x signal intensities. Experiments were then set up to detect the seven non-null combinations of analyte occurrences. Tables 18-24 provide the PCR reaction components for each of these mixtures.

Table 18. Binary FAM Experiment 1 Cocktail: HIV Polyprotease, HIV p17, and dengue virus

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Reagents	Concentration	Volume Added
UltraPure Water	-	50µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	4µL
P17 Template	15nM	4µL
Dengue Virus Template	17nM	4µL
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 19. Binary FAM Experiment 2 Cocktail: dengue virus and HIV p17

Reagents	Concentration	Volume Added
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	-
P17 Template	15nM	4µL
Dengue Virus Template	17nM	4µL
Primers	Concentration	Volume Added

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Poly Protease FWD Primer	1μM	4μL
Poly Protease RVS Primer	1μM	4μL
P17 FWD Primer	1μM	4μL
P17 RVS Primer	1μM	4μL
Dengue Virus FWD Primer	1μM	4μL
Dengue Virus RVS Primer	1μM	4μL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4μL
P17 Probe 1	400nM	4μL
Dengue Virus Probe 1	200nM	4μL

Table 20. Binary FAM Experiment 3 Cocktail: dengue virus and HIV polyprotease

Reagents	Concentration	Volume Added
UltraPure Water	-	54μL
Taq 5x Master Mix	-	25μL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	4μL
P17 Template	15nM	-
Dengue Virus Template	17nM	4μL
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1μM	4μL
Poly Protease RVS Primer	1μM	4μL
P17 FWD Primer	1μM	4μL
P17 RVS Primer	1μM	4μL
Dengue Virus FWD Primer	1μM	4μL
Dengue Virus RVS Primer	1μM	4μL

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Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 21. Binary FAM Experiment 4 Cocktail: HIV polyprotease and HIV p17

Reagents	Concentration	Volume Added
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	4µL
P17 Template	15nM	4µL
Dengue Virus Template	17nM	-
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 22. Binary FAM Experiment 5 Cocktail: dengue virus

Reagents	Concentration	Volume Added
UltraPure Water	-	58µL

Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	-
P17 Template	15nM	-
Dengue Virus Template	17nM	4µL
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 23. Binary FAM Experiment 6 Cocktail: HIV p17

Reagents	Concentration	Volume Added
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	-
P17 Template	15nM	4µL
Dengue Virus Template	17nM	-
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL

P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 24. Binary FAM Experiment 7 Cocktail: HIV polyprotease

Reagents	Concentration	Volume Added
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	4µL
P17 Template	15nM	-
Dengue Virus Template	17nM	-
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

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[00188] The total fluorescence signal of each experiment was plotted on a chromatogram (**Fig. 7**) that was constructed as described above. The width of the bands around each positive control measurement is the propagated uncertainty of the 1X measurement. That uncertainty was equated to the standard deviation of the fluorescence signals of the last five PCR cycles in saturation.

[00189] **Fig. 7** shows the results of this experiment. The three right-most results show the measured intensities for dengue virus alone (DEN), HIV p17 alone (P17), and HIV polyprotease (TPP). The assigned intensities for each of these analytes were as indicated in Table 11, and **Fig. 7** confirms that the three analytes yielded the expected intensity results when alone (see DEN, P17, and TPP). At the end of the assay, essentially all of the probes have been hydrolyzed, releasing essentially all of the fluorophores, which emit a signal. A fixed amount of a fluorophore produces a fixed and predictable amount of signal. Therefore a target that is present should generally produce a fixed amount of signal, as determined by the amount of probe added to the reaction volume.

[00190] As expected, the intensity values for the combinations of analytes are equivalent to the sum of the assigned intensities. Since this coding scheme is designed to be non-degenerate, each result can be unambiguously decoded into the presence or absence of a particular analyte. For example, starting from the left side of **Fig. 7**, a result of 7 indicates the presence of all three analytes. A result of 3 indicates the presence of dengue virus and HIV p17 analytes, and the absence of HIV polyprotease. A result of 5 indicates the presence of dengue virus and HIV polyprotease, and the absence of HIV p17. Finally a result of 6 indicates the presence of HIV p17 and HIV polyprotease, and the absence of dengue virus. This coding scheme can be extended to encode additional analytes, as described elsewhere in this disclosure.

Supplemental Tables and Figures

Table S1 HIV-1 Poly Protease Sequence Information

Sequence Information	HIV-1 Poly protease 198mer synthesized from bases 2253 – 2550
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (5' to 3')	GGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGA GTTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTT

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	ATCAAAGTAAGACAGTATGATCAGATACTCATAGAAATCTGTGGACATAA AGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGG
Forward Primer (5' to 3')	GGAAGCTCTATTAGATACAGGAGCAG
Reverse Primer (5' to 3')	CCAATTATGTTGACAGGTGTAGGTCC
Probe 1 (5' to 3')	/**56-FAM**/TGAGTTTGCCAGGAAGATGGAAACCA/3BHQ_1/

Table S2 HIV-1 P17 Sequence Information

Sequence Name	HIV-1 P17 199mer synthesized from bases 790 – 1186
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (5' to 3')	CAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATA TAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAG ACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAG AAAAAAGCACAGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGTCA
Forward Primer (5' to 3')	CAGCTACAACCATCCCTTCAGACA
Reverse Primer (5' to 3')	TGACCTGATTGCTGTGTCCTGTGT
Probe 1 (5' to 3')	/**56-FAM**/AGCAACCCTCTATTGTGTGCATCAAAGG/3BHQ_1
Probe 2 (5' to 3')	/**5Cy3**/AAAGCACAGCAAGCAGCAGCTGA/3BHQ_2/

Table S3 Malaria ChR7 Sequence Information

Sequence Name	Malaria ChR7 199mer synthesized from bases 1139138 - 1141223
Source	UCSC Plasmodium falciparum Genome Browser Gateway
Target sequence (5' to 3')	GCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAG AGGGAGCCTGAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTA AATTACCCAATTCTAAAGAAGAGAGGTAGTGACAAGAAATAACAATGCAA GGCCAATTTAAAACCTTCCCAGAGTAACAATTGGAGGGCAAGTCTGGTG
Forward Primer (5' to 3')	GCCTAACATGGCTATGACGGGTAA
Reverse Primer (5' to 3')	CACCAGACTTGCCCTCCAATTGTT
Probe 1 (5' to 3')	/**56-FAM**/ATTCCGGAGAGGGAGCCTGAGAAATA/3BHQ_1/
Probe 2 (5' to 3')	/**56-ROXN**/AAGGAAGGCAGCAGGCGCGTAAATTA/3BHQ_2/

Table S4 Herpes Simplex Virus-2 Sequence Information

Sequence Name	HSV-2 193mer synthesized from HSV-2 genome
Source	EMBL Bank AJ303204
Target sequence (5' to 3')	TCAGCCCATCCTCCTTCGGCAGTATGGAGGGTGTCGCGGCGGCGAGCCGC CGTCCCCAAAGACGTGCGGGTCGTACACGTACACGTACCAGGGCGGCGGG

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	CCTCCGACCCGGTACGCTCTCGTAAATGCTTCCCTGCTGGTGCCGATCTG GGACCGCGCCGCGGAGACATTCGAGTACCAGATCGAACTCGG
Forward Primer (5' to 3')	TCAGCCCATCCTCCTTCGGCAGTAT
Reverse Primer (5' to 3')	CCGAGTTCGATCTGGTACTCGAATGT
Probe 1 (5' to 3')	/**56-FAM**/AAAGACGTGCGGGTCGTACACGTACA/3BHQ_1/
Probe 2 (5' to 3')	/**5Cy5**/TAAATGCTTCCCTGCTGGTGCCGAT/3IAbRQSp/

Table S5 Tuberculosis rpoB Sequence Information

Sequence Name	Mycobacterium Tuberculosis rpoB 200mer synthesized from Mycobacterium tuberculosis rpoB genome
Source	EMBL Bank GQ395623
Target sequence (5' to 3')	GAGTGCAAAGACAAGGACATGACGTACGCGGCCCCGCTGTTCGTCACGGC CGAGTTCATCAACAACAACACCGGCGAGATCAAGAGCCAGACGGTGTTCA TGGGTGACTTCCCGATGATGACCGAGAAGGGCACCTTCATCATCAACGGC ACCGAGCGCGTCGTGGTCAGCCAGCTGGTCCGCTCGCCCGGTGTGTACTT
Forward Primer (5' to 3')	GAGTGCAAAGACAAGGACATGACG
Reverse Primer (5' to 3')	AAGTACACACCGGGCGAGC
Probe 1 (5' to 3')	/**56-FAM**/CGCTGTTCGTCACGGCCGAGTTCAT/3BHQ_1/
Probe 2 (5' to 3')	/**5Cy3**/AGATCAAGAGCCAGACGGTGTTCATG/3BHQ_2/
Probe 3 (5' to 3')	/**5Cy5**/AAGGGCACCTTCATCATCAACGGCA/3IAbRQSp/

Table S6 Dengue Virus Type 3 Sequence Information

Sequence Name	Dengue Virus Type 3 200mer synthesized from Dengue Virus Type 3 genome
Source	GenBank M93130
Target sequence (5' to 3')	ATGCCAACTGTGATTGAGCACTTAGAAAGACTACAAAGGAAACATGGAGG AATGCTTGTGAGAAATCCACTCTCACGAAACTCCACGCACGAAATGTATT GGATATCCAATGGTACAGGCAACATCGTCTCATTCACAATGACACACAGG AGACCCACCATAGAGAAAGATGTGGATTTAGGAGCAGGAACCCGACATGT
Forward Primer (5' to 3')	ATGCCAACTGTGATTGAGCACT
Reverse Primer (5' to 3')	ACATGTCGGGTTCCTGCTCCTAAA
Probe 1 (5' to 3')	/**56-FAM**/ACAAAGGAAACATGGAGGAATGCTTGTGA/3BHQ_1/
Probe 2 (5' to 3')	/**5Cy3**/ACTCTCACGAAACTCCACGCACGAAA/3BHQ_2/
Probe 3 (5' to 3')	/**56-ROXN**/ACAATGACACACAGGAGACCCACCAT/3BHQ_2/

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Probe 4 (5' to 3')	/5Cy5/GGATATCCAATGGTACAGGCAACATCGT/3IAbRQSp/

Table S7 Experiment 1 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	34µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	2µL
Poly Protease RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1µM	2µL

Table S8 Experiment 2 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	28µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Malaria Template	15nM	2µL
Herpes Template	17nM	2µL
TB Template	16nM	2µL
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	2µL
Poly Protease RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1µM	2µL

Table S9 Experiment 3 Cocktail

Reagents	**Concentration**	**Volume Added**

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UltraPure Water	-	32µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Malaria Template	15nM	2µL
Primers	**Concentration**	**Volume Added**
Malaria FWD Primer	1µM	2µL
Malaria RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Malaria Probe 1	1µM	2µL
Malaria Probe 2	1µM	2µL

Table S10 Experiment 4 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	26µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Herpes Template	17nM	2µL
TB Template	16nM	2µL
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Malaria FWD Primer	1µM	2µL
Malaria RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Malaria Probe 1	1µM	2µL
Malaria Probe 2	1µM	2µL

Table S11 Experiment 5 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	32µL
Taq 5x Master Mix	-	42µL

Templates	Concentration	Volume Added
Herpes Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Herpes FWD Primer	1µM	2µL
Herpes RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Herpes Probe 1	1µM	2µL
Herpes Probe 2	1µM	2µL

Table S12 Experiment 6 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	26µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Malaria Template	15nM	2µL
TB Template	16nM	2µL
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Herpes FWD Primer	1µM	2µL
Herpes RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Herpes Probe 1	1µM	2µL
Herpes Probe 2	1µM	2µL

Table S13 Experiment 7 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	30µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
TB Template	16nM	2µL

Primers	Concentration	Volume Added
TB FWD Primer	1µM	2µL
TB RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
TB Probe 1	1µM	2µL
TB Probe 2	1µM	2µL
TB Probe 3	1µM	2µL

Table S14 Experiment 8 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	30µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Malaria Template	15nM	2µL
Herpes Template	17nM	2µL
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
TB FWD Primer	1µM	2µL
TB RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
TB Probe 1	1µM	2µL
TB Probe 2	1µM	2µL
TB Probe 3	1µM	2µL

Table S15 Experiment 9 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	28µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Dengue Virus Template	17nM	2µL

Primers	Concentration	Volume Added
Dengue Virus FWD Primer	1µM	2µL
Dengue Virus RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Dengue Virus Probe 1	1µM	2µL
Dengue Virus Probe 2	1µM	2µL
Dengue Virus Probe 3	1µM	2µL
Dengue Virus Probe 4	1µM	2µL

Table S16 Experiment 10 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	22µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Malaria Template	15nM	2µL
Herpes Template	17nM	2µL
TB Template	16nM	2µL
Primers	**Concentration**	**Volume Added**
Dengue Virus FWD Primer	1µM	2µL
Dengue Virus RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Dengue Virus Probe 1	1µM	2µL
Dengue Virus Probe 2	1µM	2µL
Dengue Virus Probe 3	1µM	2µL
Dengue Virus Probe 4	1µM	2µL

Table S17 Experiment 11 Cocktail

Reagents	Concentration	Volume Added
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**

Poly Protease Template	17nM	2µL
Malaria Template	15nM	2µL
Herpes Template	17nM	2µL
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	2µL
Poly Protease RVS Primer	1µM	2µL
Malaria FWD Primer	1µM	2µL
Malaria RVS Primer	1µM	2µL
Herpes FWD Primer	1µM	2µL
Herpes RVS Primer	1µM	2µL
Dengue Virus FWD Primer	1µM	2µL
Dengue Virus RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1µM	2µL
Malaria Probe 1	1µM	2µL
Malaria Probe 2	1µM	2µL
Herpes Probe 1	1µM	2µL
Herpes Probe 2	1µM	2µL
Dengue Virus Probe 1	1µM	2µL
Dengue Virus Probe 2	1µM	2µL
Dengue Virus Probe 3	1µM	2µL
Dengue Virus Probe 4	1µM	2µL

Table S18 Experiment 12 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	2µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Malaria Template	15nM	2µL
Herpes Template	17nM	2µL

TB Template	16nM	2µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	2µL
Poly Protease RVS Primer	1µM	2µL
Malaria FWD Primer	1µM	2µL
Malaria RVS Primer	1µM	2µL
Herpes FWD Primer	1µM	2µL
Herpes RVS Primer	1µM	2µL
TB FWD Primer	1µM	2µL
TB RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1µM	2µL
Malaria Probe 1	1µM	2µL
Malaria Probe 2	1µM	2µL
Herpes Probe 1	1µM	2µL
Herpes Probe 2	1µM	2µL
TB Probe 1	1µM	2µL
TB Probe 2	1µM	2µL
TB Probe 3	1µM	2µL

Table S19 Experiment 13 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	12µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Herpes Template	17nM	2µL
TB Template	16nM	2µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	2µL
Poly Protease RVS Primer	1µM	2µL
Herpes FWD Primer	1µM	2µL

Herpes RVS Primer	1µM	2µL
TB FWD Primer	1µM	2µL
TB RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1µM	2µL
Herpes Probe 1	1µM	2µL
Herpes Probe 2	1µM	2µL
TB Probe 1	1µM	2µL
TB Probe 2	1µM	2µL
TB Probe 3	1µM	2µL

Table S20 Experiment 14 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	10µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Malaria Template	15nM	2µL
Dengue Virus Template	17nM	2µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	2µL
Poly Protease RVS Primer	1µM	2µL
Malaria FWD Primer	1µM	2µL
Malaria RVS Primer	1µM	2µL
Dengue Virus FWD Primer	1µM	2µL
Dengue Virus RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1µM	2µL
Malaria Probe 1	1µM	2µL
Malaria Probe 2	1µM	2µL
Dengue Virus Probe 1	1µM	2µL
Dengue Virus Probe 2	1µM	2µL

Dengue Virus Probe 3	1µM	2µL
Dengue Virus Probe 4	1µM	2µL

Table S21 Binary FAM Experiment 1 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	50µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	4µL
P17 Template	15nM	4µL
Dengue Virus Template	17nM	4µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table S22 Binary FAM Experiment 2 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	-
P17 Template	15nM	4µL
Dengue Virus Template	17nM	4µL
Primers	**Concentration**	**Volume Added**

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Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table S23 Binary FAM Experiment 3 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	4µL
P17 Template	15nM	-
Dengue Virus Template	17nM	4µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table S24 Binary FAM Experiment 4 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	4µL
P17 Template	15nM	4µL
Dengue Virus Template	17nM	-
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table S25 Binary FAM Experiment 5 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	-
P17 Template	15nM	-
Dengue Virus Template	17nM	4µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL

Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table S26 Binary FAM Experiment 6 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	-
P17 Template	15nM	4µL
Dengue Virus Template	17nM	-
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table S27 Binary FAM Experiment 7 Cocktail

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**

Poly Protease Template	17nM	4µL
P17 Template	15nM	-
Dengue Virus Template	17nM	-
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

PATENT
Attorney Docket No.: 88DG-373535-US
CIT 6088-C-C-C3-C2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:		Group Art Unit:	1672
	Emil P. KARTALOV *et al.*	Examiner:	Russell Scott NEGIN
Serial No.:	18/352,112	Conf. No.	3145
For:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	***VIA Patent Center***	

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

RESPONSE TO NOTICE TO FILE CORRECTED APPLICATION PAPERS
(*Notice of Allowance Mailed*)

In response to the Notice to File Corrected Application Papers ("Notice") dated August 1, 2024, the period for response extending through October 1, 2024, Applicant submits the following:

1. A Substitute Specification (clean) is being submitted to remove table shadings and colored fonts at pages 76-89, in compliance with 37 CFR 1.52 and the exceptions of 37 CFR 1.58(a). Further, illustrations on pages 90-91 have been removed, in compliance with 37 CFR 1.52 and the exceptions of 37 CFR 1.58(a); and

2. One replacement drawing sheet for FIG. 8 (in grey scale) is being submitted providing clarification changes in the illustrations on pages 90-91 to FIG. 8.

No new matter is added. A copy of the Notice accompanies this reply.

Applicant believes that no fee is required for submitting this response. If any fee(s) is required, the Commissioner is hereby authorized to charge the required fee(s) which may be due to Deposit Account 50-4561.

Dated: September 19, 2024

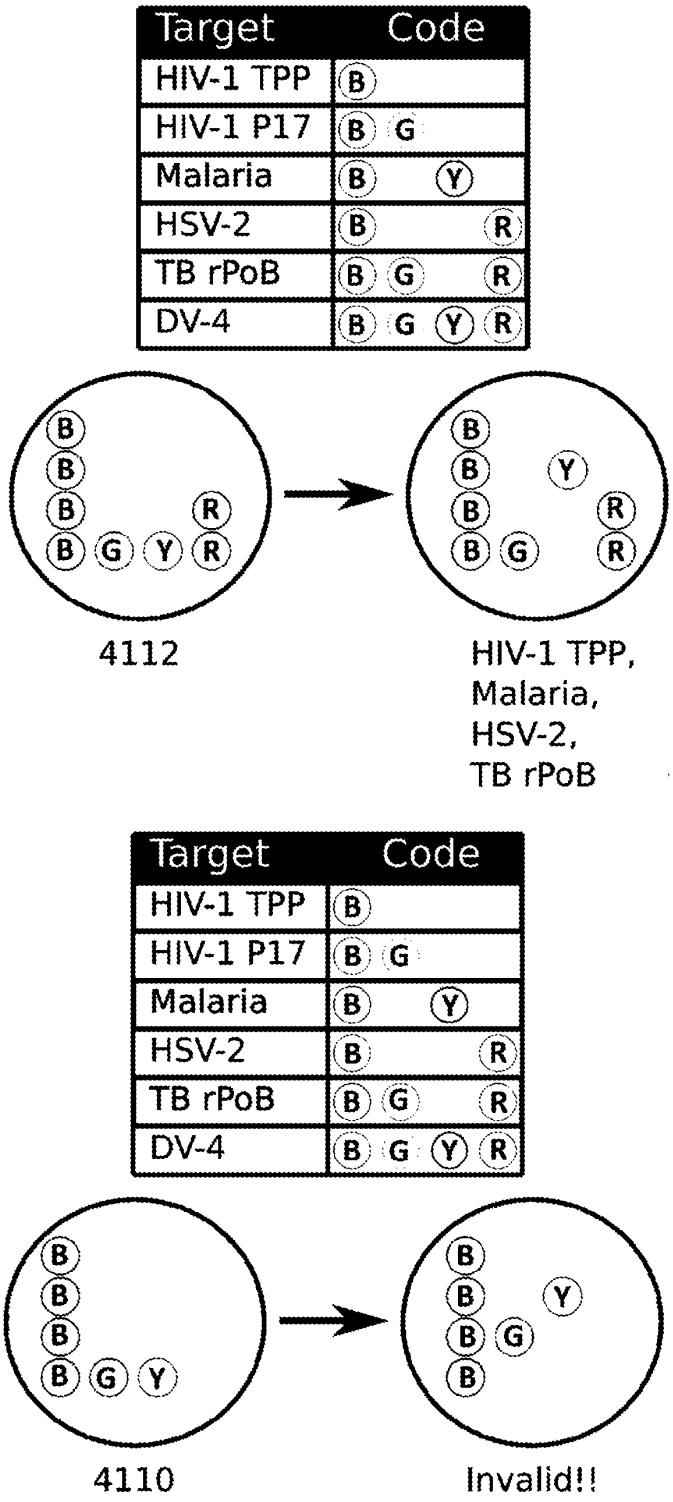
Respectfully submitted,

By /David E. Heisey/
David E. Heisey
Reg. No. 42651

SHEPPARD MULLIN RICHTER & HAMPTON LLP
12275 El Camino Real, Suite 100
San Diego, CA 92130
Customer Number: 30764
Attorney for Applicant
(858) 720-8900

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Fig. 8


Target
Code
HIV-1 TPP
B
HIV-1 P17
B G
Malaria
B Y
HSV-2
B R
TB rPoB
B G R
DV-4
B G Y R
4112
HIV-1 TPP,
Malaria,
HSV-2,
TB rPoB
Target
Code
HIV-1 TPP
B
HIV-1 P17
B G
Malaria
B Y
HSV-2
B R
TB rPoB
B G R
DV-4
B G Y R
4110
Invalid!!

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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/352,112	**09/19/2024 05:24:09 PM Z ET**	**88DG-373535-US**

Title of Invention

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	3145	FILED BY	Annie Wong
PATENT CENTER #	67247018	FILING DATE	07/13/2023
CUSTOMER #	30764	FIRST NAMED INVENTOR	Emil P. KARTALOV
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	David Heisey

Documents

TOTAL DOCUMENTS: 4

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
NTFCAP_Response-after_NOA_18352112.pdf	2	Post Allowance Communication - Incoming	24 KB
Replacement_Drawing_18352112.pdf	1	Drawings-only black and white line drawings	192 KB
Copy_NTFCAP_18352112.pdf	3	Post Allowance Communication - Incoming	99 KB
Substitute_Spec-clean_18352112.pdf	93	Specification	488 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)

NTFCAP_Response-after_NOA_18352112.pdf	757A789659AA2B71B47B6035DB93EDAE7ED4EE2CD7E6846FCC71039A5E2DE79AA3F3EE43B61C2C9161037692ED8721B227F51738FE7D8197B1B6600E1C941906
Replacement_Drawing_18352112.pdf	A9936B47F0643CC0643DF1A8473C548EE26DEF3101FDF40235AC6AE6FB94E6342DE0DE353525AD06A03E23DE7376272498DA361251BCD99D36359C9FB82F9916
Copy_NTFCAP_18352112.pdf	964E453ABAE89DDE269822BBFF83F27D77986A071D7AA259945E624228635C6F2FECB1C7E3FAC2BBAD912B8DD062396F05912670503CFD16B8F8F316FD46DE51
Substitute_Spec-clean_18352112.pdf	130719A9433E784E02E32480AA88499B0179685EDEB535367BAE1A7C3B0AA796EC34F4DCCB73E4DDA545DB291E73BCDE5DE88B60EEA4E0A07BB730D4443A6F70

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 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.

DocCode - SCORE

Placeholder Sheet for Supplemental Content

Application Number: 18352112 Document Date: 09/19/2024

The presence of this form indicates that the following document type was received in electronic format on the date identified above. This document is stored separately from the typical case contents which are stored in image format.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded and no paper documents or physical media exist.

- Drawing

At the time of document entry (noted above):

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Form Revision Date: March 1, 2024

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/352,112	07/13/2023	Emil P. KARTALOV	88DG-373535-US	3145

30764 7590 09/17/2024

Sheppard, Mullin, Richter & Hampton LLP
650 Town Center Drive, 10th Floor
Costa Mesa, CA 92626

EXAMINER
NEGIN, RUSSELL SCOTT

ART UNIT	PAPER NUMBER
1672	

NOTIFICATION DATE	DELIVERY MODE
09/17/2024	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):

SheppardMullin_Pair@firsttofile.com
docketing@sheppardmullin.com

Notice of Allowability	Application No. 18/352,112	Applicant(s) KARTALOV et al.	
	Examiner RUSSELL S NEGIN	Art Unit 1672	AIA (FITF) Status No

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

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to the communications of 7/24/2024 and 8/29/2024.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

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

3. ☑ The allowed claim(s) is/are 1-30 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

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

   Certified copies:

   a) ☐ All b) ☐ Some* c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☐ Examiner's Amendment/Comment
6. ☐ Examiner's Statement of Reasons for Allowance
7. ☑ Other PTO-271 form.

/RUSSELL S NEGIN/
Primary Examiner, Art Unit 1672

IPR2025-01546
California Institute of Technology - Ex. 2026, Page 395

Response to Rule 312 Communication	Application No.	Applicant(s)	
	18/352,112	KARTALOV et al.	
	Examiner	Art Unit	AIA (FITF) Status
	RUSSELL S NEGIN	1672	No

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

1. ☑ The amendment filed on 29 August 2024 under 37 CFR 1.312 has been considered, and has been:
   - a) ☑ entered.
   - b) ☐ entered as directed to matters of form not affecting the scope of the invention.
   - c) ☐ disapproved because the amendment was filed after the payment of the issue fee.
     Any amendment filed after the date the issue fee is paid must be accompanied by a petition under 37 CFR 1.313(c)(1) and the required fee to withdraw the application from issue.
   - d) ☐ disapproved. See explanation below.
   - e) ☐ entered in part. See explanation below.
   - f) ☐ not entered because the supplemental or corrected Application Data sheet (ADS)
     - ☐ was not accompanied by a petition to accept an unintentionally delayed claim under 37 CFR 1.55 or 27 CFR 1.78;
     - ☐ did not identify the information being changed in accordance with 37 CFR 1.76(c)(2);
     - ☐ was not properly signed in accordance with 37 CFR 1.76(e) (or 37 CFR 1.33(b) for applications filed prior to September 16, 2012).

/RUSSELL S NEGIN/
Primary Examiner, Art Unit 1672

PATENT
Docket No.: 88DG-373535-US
CIT 6088-C-C-C3-C2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Emil P. KARTALOV *et al.*	Allowed: July 8, 2024
Application No.: 18,352,112	Confirmation No.: 3145
Filed: July 13, 2023	Art Unit: 1672
For: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	Examiner: Russell Scott NEGIN

AMENDMENT AFTER ALLOWANCE UNDER 37 C.F.R. § 1.312

OK TO ENTER
9/13/2024 /RSN/

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

Dear Commissioner:

INTRODUCTORY COMMENTS

Prior to issuance of the patent, applicant respectfully requests entry of this amendment under 37 C.F.R. 1.312 for the above-captioned patent application.

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

Remarks follow the amendments section of this paper.

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

PTO/SB/08a (01-10)
Approved for use through 07/31/2012. OMB 0651-0031
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	18352112
Filing Date	2023-07-13
First Named Inventor	Emil P. KARTALOV
Art Unit	1672
Examiner Name	Russell Scott NEGIN
Attorney Docket Number	88DG-373535-US

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/RSN/	1	2011/100604	WO	A2	2011-08-18	RainDance Technologies, Inc.		☐

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/RUSSELL S NEGIN/ 09/13/2024

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	18352112
	Filing Date	2023-07-13
	First Named Inventor	Emil P. KARTALOV
	Art Unit	1672
	Examiner Name	Russell Scott NEGIN
	Attorney Docket Number	88DG-373535-US

/RSN/	1	Extended European Search Report dated June 20, 2024, issued in related European Patent Application No. 24161841.2 (25 pages).	☐
/RSN/	2	Extended European Search Report dated June 18, 2024, issued in related European Patent Application No. 24161820.6 (13 pages).	☐
/RSN/	3	Extended European Search Report dated June 17, 2024, issued in related European Patent Application No. 24161844.6 (15 pages).	☐
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CC: patentcenter_eofficeaction@uspto.gov
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Tue Sep 17 05:00:59 EDT 2024

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Application	Document	Mailroom Date	Attorney Docket No.
18352112	NOA	09/17/2024	88DG-373535-US
18352112	N271	09/17/2024	88DG-373535-US
18352112	1449	09/17/2024	88DG-373535-US

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APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/352,112	**08/29/2024 04:32:34 PM Z ET**	**88DG-373535-US**

Title of Invention

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	3145	FILED BY	Annie Wong
PATENT CENTER #	66982500	FILING DATE	07/13/2023
CUSTOMER #	30764	FIRST NAMED INVENTOR	Emil P. KARTALOV
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	David Heisey

Documents

TOTAL DOCUMENTS: 3

DOCUMENT		PAGES	DESCRIPTION	SIZE (KB)
312_Amendment_18352112.pdf		10	-	96 KB
312_Amendment_18352112-A.NA.pdf	(1-1)	1	Amendment after Notice of Allowance (Rule 312)	18 KB
312_Amendment_18352112-CLM.pdf	(2-9)	8	Claims	44 KB
312_Amendment_18352112-REM.pdf	(10-10)	1	Applicant Arguments/Remarks Made in an Amendment	52 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)

312_Amendment_18352112.pdf	FCC98937BC52DACC03E5AB2D579003C32DB10FE4C67CC51A5E2ED274FD4A9840C027248D21EDE5CE11D776CFFBDD75AFE5F9051396C57387B81BB70015BA7668
312_Amendment_18352112-A.NA.pdf	55EBF3FD01E87FF0EDF04D9044474303C39D2BD0BAB787A31CF7A12667C722BF643BA99101DE06326E8ED785D3B13FD01434CEC3696A50C5B5B5478F5F60C644
312_Amendment_18352112-CLM.pdf	C37BB3421152F1A1E091A2D66381A42A5105A6082F39C8D4AA4909C177174F66257CF4AB49942F9F9C729C612FD79E6797A72097BAA3CD1A8838EDC71B85EE5E
312_Amendment_18352112-REM.pdf	0F4DF8C94DF4D7767AD1C9C3A833394872EAE7361648E07E4BB15518AB66BEA1C66FA8A08F53C6BEFE60577A82B7A027D9CA64E2A2629174CA433FBE979A30D9

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PATENT
Docket No.: 88DG-373535-US
CIT 6088-C-C-C3-C2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Emil P. KARTALOV *et al.*	Allowed: July 8, 2024
Application No.: 18,352,112	Confirmation No.: 3145
Filed: July 13, 2023	Art Unit: 1672
For: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	Examiner: Russell Scott NEGIN

AMENDMENT AFTER ALLOWANCE UNDER 37 C.F.R. § 1.312

MS Issue Fee
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Dear Commissioner:

INTRODUCTORY COMMENTS

Prior to issuance of the patent, applicant respectfully requests entry of this amendment under 37 C.F.R. 1.312 for the above-captioned patent application.

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

Remarks follow the amendments section of this paper.

AMENDMENTS TO THE CLAIMS:

The following listing of claims will replace all prior versions and listings of claims in the Application.

1. (Currently Amended) A system comprising:

a sample chamber configured to house a sample and analyte-specific reagent mixtures of analyte-specific hybridization probes and multiple fluorophores;

a multi-channel detector to detect:

a first electromagnetic signal at a first wavelength from the sample chamber, the first electromagnetic signal generated by excitement of a first fluorophore of the multiple fluorophores;

a second electromagnetic signal at a second wavelength from the sample chamber, the second electromagnetic signal generated by excitement of a second fluorophore of the multiple fluorophores;

a third electromagnetic signal at a third wavelength from the sample chamber, the third electromagnetic signal generated by excitement of a third fluorophore of the multiple fluorophores;

a fourth electromagnetic signal at a fourth wavelength from the sample chamber, the fourth electromagnetic signal generated by excitement of a fourth fluorophore of the multiple fluorophores;

a processor controlled analyzer to receive, from the multi-channel detector, a cumulative signal based on the first, second, third, and fourth electromagnetic signals and apply a decoding matrix to the cumulative signal to unambiguously detect the presence or absence of at least each of M analytes by associating, for each analyte, a first value in a first component of the cumulative signal and a second value in a second component of the cumulative signal, wherein each first value is an intensity or range of intensities and each second value is a wavelength or a range of wavelengths, and wherein the second values comprise the first, second, third, and fourth wavelengths, and the determination is made without immobilization, mass spectrometry or melting curve analysis;

wherein for the positive integer M,

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M=C*log2 (F+1),

F is a positive integer and is equal to the maximum cumulative intensity of the first component of the signal, for any second value, when all of the analytes are present, and

C=4, 5, or 6; and

wherein F+1 is a positive integer and wherein F+1 is ~~1 or~~ a power of 2.

wherein M is greater than the number of the second values used to encode the analytes (C), the multi-channel detector comprises C channels, and M and C are positive integers[[;]].

2. (Previously Presented) The system of claim 1, wherein the multi-channel detector is further configured to detect:

a fifth electromagnetic signal at a fifth wavelength from the sample chamber, the fifth electromagnetic signal generated by excitement of a fifth fluorophore of the multiple fluorophores;

a sixth electromagnetic signal at a sixth wavelength from the sample chamber, the sixth electromagnetic signal generated by excitement of a sixth fluorophore of the multiple fluorophores; and

wherein C= 6.

3. (Previously Presented) The system of claim 1, wherein F=1, 3, 7, 15, 31, 63, or 127.

4. (Previously Presented) The system of claim 1, wherein F=3.

5. (Previously Presented) The system of claim 4, wherein the multiple fluorophores is selected from the group consisting of a blue fluorophore, a green fluorophore, a yellow fluorophore, a red fluorophore, and any combinations thereof.

6. (Previously Presented) The system of claim 4, wherein the multiple fluorophores is selected from the group consisting of a fluorescein amidite (FAM), a cyanine 3 (Cy3), a carboxy-X-rhodamine (ROX), a cyanine 5 (Cy5), a cyanine 5.5 (Cy5.5), and any combinations thereof.

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7. (Previously Presented) The system of claim 4, wherein the multiple fluorophores has a maximum excitation wavelength selected from the group consisting of about 494 nm, about 550 nm, about 567 nm, about 650 nm, about 675 nm, and any combinations thereof.

8. (Previously Presented) The system of claim 4, wherein the multiple fluorophores has a maximum emission wavelength selected from the group consisting of about 518 nm, about 565 nm, about 591 nm, about 670 nm, about 697 nm, and any combinations thereof.

9. (Previously Presented) The system of claim 4, wherein the analyte-specific hybridization probes are labeled with a fluorophore of the multiple fluorophores.

10. (Previously Presented) The system of claim 4, wherein the detector comprises four or six band pass filters.

11. (Previously Presented) The system of claim 4, wherein the detector comprises band pass filters that enable the detection of blue, green, and red fluorescent signals; and wherein the detector comprises band pass filters that enable the detection of ranges selected from the group consisting of from about 483 nm to about 533 nm, from about 523 nm to about 568 nm, from about 558 nm to about 610 nm, from about 615 nm to about 670 nm, and any combinations thereof.

12. (Previously Presented) The system of claim 4, wherein the multi-channel detector comprises four or six photodetectors.

13. (Previously Presented) The system of claim 4, wherein the multi-channel detector comprises photodetectors that enable the detection of blue, green, and red fluorescent signals.

14. (Previously Presented) The system of claim 4, wherein the multi-channel detector comprises photodetectors that enable the detection ranges selected from the group consisting of

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from about 483 nm to about 533 nm, from about 523 nm to about 568 nm, from about 558 nm to about 610 nm, from about 615 nm to about 670 nm, and any combinations thereof.

15. (Previously Presented) The system of claim 4, wherein the sample comprises a droplet.

16. (Previously Presented) The system of claim 15, wherein the droplet is housed in a dynamic array.

17. (Previously Presented) The system of claim 2, wherein each of the six fluorophores is attached to a hybridization probe.

18. (Previously Presented) The system of claim 4, further comprising a display coupled to the processor controlled analyzer to visualize a plot of the first and second values.

19. (Currently Amended) A system comprising:

a processor;

a display; and

a non-transitory computer readable medium storing instructions thereon that, when executed by the processor, cause the processor to:

obtain cumulative signal data from a digital PCR instrument with a sample volume, the digital PCR instrument comprising a light source and a multichannel detector with C channels, and the sample volume comprising M fluorescently labeled polynucleotide analytes;

apply a decoding matrix to the cumulative signal data to unambiguously determine the presence or absence of at least each of the M fluorescently labeled polynucleotide analytes by associating, for each fluorescently labeled polynucleotide analyte, a first value in a first component of the cumulative signal data and a second value in a second component of the cumulative signal data, wherein each first value is an intensity or range of intensities and each second value is a wavelength or a range of wavelengths,

wherein for a positive integer M,

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$M=C*\log_2(F+1)$,

F is the maximum cumulative intensity of the first component of the cumulative signal data, for any second value, when all of the analytes are present,

M, C, and F are each positive integers, and

wherein F+1 is a positive integer and wherein F+1 is ~~1 or~~ a power of 2; and

plot, on the display, a representation of the first value and the second value;

wherein the cumulative signal data comprises:

a first electromagnetic signal at a first wavelength from the sample volume, the first electromagnetic signal generated by excitement of a first fluorescently labeled polynucleotide analyte of the six fluorescently labeled polynucleotide analytes;

a second electromagnetic signal at a second wavelength from the sample volume, the second electromagnetic signal generated by excitement of a second fluorescently labeled polynucleotide analyte of the six fluorescently labeled polynucleotide analytes;

a third electromagnetic signal at a third wavelength from the sample volume, the third electromagnetic signal generated by excitement of a third fluorescently labeled polynucleotide analyte of the six fluorescently labeled polynucleotide analytes;

a fourth electromagnetic signal at a fourth wavelength from the sample volume, the fourth electromagnetic signal generated by excitement of a fourth first fluorescently labeled polynucleotide analyte of the six fluorescently labeled polynucleotide analytes; and

wherein the second values comprise the first, second, third, and fourth wavelengths, and the determination is made without immobilization, mass spectrometry or melting curve analysis.

20. (Currently Amended) A reaction mixture for unambiguously detecting a presence or absence of M non-immobilized polynucleotide analytes of a single sample, the reaction mixture comprising:

a plurality of non-immobilized hybridization probes,

wherein the reaction mixture is subjected to conditions such that the plurality of polynucleotide analytes are amplified;

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wherein at least one of the plurality of oligonucleotide primers is used to amplify a region complementary of at least one of the plurality of hybridization probes;

wherein the plurality of hybridization probes comprise a first hybridization probe conjugated to a first fluorophore and a second fluorophore,

wherein the first fluorophore and the second fluorophore, when excited by a light source, emit light of different wavelengths, and

the plurality of hybridization probes further comprises a third hybridization probe and a fourth hybridization probe, wherein the third hybridization probe is conjugated to a third fluorophore and the fourth hybridization is conjugated to a fourth fluorophore,

wherein the third fluorophore and the fourth fluorophore, when excited by a light source, emit light of a same wavelength, and

wherein the third hybridization probe and the fourth hybridization probe are present at different concentrations in the reaction mixture;

optionally the plurality of hybridization probes further comprises a fifth hybridization probe and a sixth hybridization probe, wherein the fifth hybridization probe is conjugated to a fifth fluorophore and the sixth hybridization is conjugated to a sixth fluorophore;

wherein the fifth fluorophore and the sixth fluorophore, when excited by a light source, emit light of a same wavelength, and

wherein the fifth hybridization probe and the six hybridization probe are present at different concentrations in the reaction mixture,

wherein for the positive integer M,

$$M=C*\log_2(F+1),$$

F is a positive integer and is equal to the maximum cumulative intensity of the first component of the signal, for any second value, when all of the analytes are present, and

C=4, 5, or 6; and

wherein F+1 is a positive integer and wherein F+1 is ~~1 or~~ a power of 2, and

wherein M is greater than the number of the second values used to encode the analytes , the multi-channel detector comprises C channels, and M and C are positive integers.

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21. (Previously Presented) The reaction mixture of claim 20, wherein C=6 and wherein F=1, 3, 7, 15, 31, 63, or 127.

22. (Previously Presented) The reaction mixture of claim 20, wherein F=3.

23. (Previously Presented) The system of Claim 19, wherein F=1, 3, 7, 15, 31, 63, or 127.

24. (Previously Presented) The system of Claim 19, wherein F=3.

25. (Previously Presented) The reaction mixture of claim 22, wherein a ratio of concentration between the first hybridization probe and the second hybridization probe is between 0.3 and 0.7 mol/mol.

26. (Previously Presented) The reaction mixture of claim 22, wherein the reaction mixture further comprises a polymerase having 5′ to 3′ exonuclease activity and primers specific for the plurality of polynucleotide analytes.

27. (Previously Presented) The reaction mixture of claim 22, wherein the first fluorophore, the second fluorophore, the third fluorophore, the fourth fluorophore, the fifth fluorophore, or the sixth fluorophore comprise multiple fluorophores.

28. (Previously Presented) The reaction mixture of claim 22, wherein one of the plurality of hybridization probes further comprise a fluorescent quencher.

29. (Previously Presented) The reaction mixture of claim 22, wherein the first hybridization probe and the second hybridization probe are present at different concentrations in the reaction mixture.

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30. (Previously Presented) A kit for the unambiguously detecting a presence or absence of a plurality of polynucleotide analytes of a single sample comprising the components claim 22 and instructions for the use of the kit.

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California Institute of Technology - Ex. 2026, Page 411

REMARKS

Claims 1, 19 and 20 are amended to correct their form by narrowing the scope of "F +1." No new matter has been added. Applicant believes no fee is due with this response. In the event the U.S. Patent and Trademark Office determines that a fee or an extension is required with this submission, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 50-4561 referencing docket no. 88DG-373535-US.

Dated: August 29, 2024

Respectfully submitted,

By: /David E. Heisey/
David E. Heisey
Registration No.: 42651
Attorney for Applicant

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/352,112	07/13/2023	Emil P. KARTALOV	88DG-373535-US	3145

30764 7590 08/01/2024

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NEGIN, RUSSELL SCOTT

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Application No. : 18352112
Applicant : Kartalov
Filing Date : 07/13/2023
Date Mailed : 08/01/2024

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Application No. 18352112

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- [X] Specification document coded SPEC dated 07/13/2023, page(s) 76-89.
- [ ] Claims document coded dated , claim(s) .
- [ ] Amendment document coded dated .
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Application	Document	Mailroom Date	Attorney Docket No.
18352112	M327	08/01/2024	88DG-373535-US

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(Not for submission under 37 CFR 1.99)

Application Number	18352112
Filing Date	2023-07-13
First Named Inventor	Emil P. KARTALOV
Art Unit	1672
Examiner Name	Russell Scott NEGIN
Attorney Docket Number	88DG-373535-US

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	Application Number	18352112
	Filing Date	2023-07-13
	First Named Inventor	Emil P. KARTALOV
	Art Unit	1672
	Examiner Name	Russell Scott NEGIN
	Attorney Docket Number	88DG-373535-US

	1	Extended European Search Report dated June 20, 2024, issued in related European Patent Application No. 24161841.2 (25 pages).	☐
	2	Extended European Search Report dated June 18, 2024, issued in related European Patent Application No. 24161820.6 (13 pages).	☐
	3	Extended European Search Report dated June 17, 2024, issued in related European Patent Application No. 24161844.6 (15 pages).	☐
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[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	18352112
	Filing Date	2023-07-13
	First Named Inventor	Emil P. KARTALOV
	Art Unit	1672
	Examiner Name	Russell Scott NEGIN
	Attorney Docket Number	88DG-373535-US

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	/David E. Heisey/	Date (YYYY-MM-DD)	2024-07-24
Name/Print	David E. Heisey	Registration Number	42651

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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Carpmaels & Ransford LLP
One Southampton Row
London WC1B 5HA
ROYAUME UNI

Date 20.06.2024

Reference	Application No./Patent No.
P087512EP	24161841.2 - 1122 / 4361608
Applicant/Proprietor	
California Institute of Technology	

Communication

The extended European search report is enclosed.

The extended European search report includes, pursuant to Rule 62 EPC, the European search report (R. 61 EPC) or the partial European search report/ declaration of no search (R. 63 EPC) and the European search opinion.

Copies of documents cited in the European search report are attached.

☒ 0 additional set(s) of copies of such documents is (are) enclosed as well.

The following have been approved:

☒ Abstract ☒ Title

☐ The Abstract was modified and the definitive text is attached to this communication.

The following figure(s) will be published together with the abstract: none

Refund of search fee
If applicable under Article 9 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

Should you wish to further prosecute this application in the examination phase, your attention is drawn to the provisions of Rule 70a EPC. An invitation to respond to the extended European search report will be issued once the date of publication of the European search report has been mentioned in the European Patent Bulletin (R. 69(1), R. 70(2) EPC).

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EUROPEAN SEARCH REPORT

Application Number
EP 24 16 1841

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	HAN M ET AL: "Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules", NATURE BIOTECHNOLOGY, NATURE PUBLISHING GROUP, US, vol. 19, no. 7, 1 July 2001 (2001-07-01), pages 631-635, XP002545495, ISSN: 1087-0156, DOI: 10.1038/90228	1-5, 8-10, 12-15	INV. G16B40/10 G16B25/20 C12Q1/6825 C12Q1/6851
Y	* abstract * * figures 1, 5-6 * * p.631 col.2 par.2 * * p.634 col.2 par.1-3 * * p.635 col.2 par.1, 3 *	11,16	
X	US 2003/148544 A1 (NIE SHUMING [US] ET AL) 7 August 2003 (2003-08-07)	1-5, 8-10, 12-15	
Y	* abstract * * paragraph [0014] - paragraph [0017] * * paragraph [0018]; figure 1 * * paragraph [0020] * * paragraph [0045] - paragraph [0047] * * paragraph [0069] - paragraph [0070] * * paragraph [0075] - paragraph [0079]; figure 6 * * [0024], [0086]-[0090]; figure 7 *	11,16	
	-/--		TECHNICAL FIELDS SEARCHED (IPC) G16B C12Q

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	11 June 2024	Werner, Andreas

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
O : non-written disclosure
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
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EUROPEAN SEARCH REPORT

Application Number
EP 24 16 1841

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X Y	US 2008/124705 A1 (KRAMER FRED R [US]) 29 May 2008 (2008-05-29) * paragraph [0002] - paragraph [0005] * * paragraph [0008] - paragraph [0016] * * paragraph [0019]; claims 13, 14, 17 * * paragraph [0032] - paragraph [0033] * * paragraph [0035] * * paragraph [0039] * * paragraph [0041] * * paragraph [0043]; example 1 * * paragraph [0048]; figure 5; example 4 * * [0051]-[0052]; figures 6a, 6b, 7, 8; examples 5, 7 * * claim 1 * -----	1-10, 12-15 11,16	
X	XU H ET AL: "Multiplexed SNP genotyping using the Qbead system: a quantum dot-encoded microsphere-based assay", NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, GB, vol. 31, no. 8, 15 April 2003 (2003-04-15), pages e43-1, XP002587000, ISSN: 1362-4962, DOI: 10.1093/NAR/GNG043 * abstract * * page 2, column 1, paragraph 2 - column 2, paragraph 2 * * page 3, column 1, paragraph 2 - column 2, paragraph 3; table 1 * * page 7, column 2, paragraph 4 * * figure 2 * -----	1,4,5,9, 10,12,15	
	-/--		TECHNICAL FIELDS SEARCHED (IPC)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	11 June 2024	Werner, Andreas

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EUROPEAN SEARCH REPORT

Application Number
EP 24 16 1841

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	LI Y ET AL: "Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes", NATURE BIOTECHNOLOGY, vol. 23, no. 7, 1 July 2005 (2005-07-01), pages 885-889, XP055010484, ISSN: 1087-0156, DOI: 10.1038/nbt1106 * abstract * * figure 1 * * p.889 col.1 par.3 * -& Li Yougen ET AL: "Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes. Supplementary Note 2.", Nature Biotechnology, 1 July 2005 (2005-07-01), pages 1-1, XP093170473, Retrieved from the Internet: URL:https://static-content.springer.com/esm/art:10.1038/nbt1106/MediaObjects/41587_2005_BFnbt1106_MOESM11_ESM.pdf [retrieved on 2024-06-04] * the whole document *	1,5,9, 10,12,15	
			TECHNICAL FIELDS SEARCHED (IPC)
X	LIN C ET AL: "Self-Assembled Combinatorial Encoding Nanoarrays for Multiplexed Biosensing", NANO LETTERS, vol. 7, no. 2, 1 February 2007 (2007-02-01), pages 507-512, XP055031573, ISSN: 1530-6984, DOI: 10.1021/nl062998n * abstract * * figure 1 * * p.508 col.1 par.1, col.2 par.2 * * p.509 col.2 l.21 *	1,5,9, 10,12	

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	11 June 2024	Werner, Andreas

CATEGORY OF CITED DOCUMENTS

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EUROPEAN SEARCH REPORT

Application Number
EP 24 16 1841

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	US 2002/146734 A1 (ORTYN WILLIAM E [US] ET AL) 10 October 2002 (2002-10-10) * abstract * * paragraph [0002] - paragraph [0003] * * paragraph [0005] * * paragraph [0014] - paragraph [0019]; figure 1 * * paragraph [0025] - paragraph [0026] * * paragraph [0028] - paragraph [0031] * * paragraph [0059] * * paragraph [0065]; figure 2C * * paragraph [0083] - paragraph [0086] * * paragraph [0092] * * paragraph [0097] - paragraph [0098]; figures 16-17 * * paragraph [0101] * -----	1,5-7,9, 10,12,15	
Y	WO 2011/100604 A2 (RAINDANCE TECHNOLOGIES INC [US]; LARSON JONATHAN WILLIAM [US] ET AL.) 18 August 2011 (2011-08-18) * the whole document * -----	11,16	
			TECHNICAL FIELDS SEARCHED (IPC)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	11 June 2024	Werner, Andreas

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ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 24 16 1841

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11-06-2024

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2003148544 A1	07-08-2003	AU 2002322348 A1	03-03-2003
		CA 2450725 A1	09-01-2003
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		JP 2023065360 A	12-05-2023
		US 2011244455 A1	06-10-2011
		US 2011250597 A1	13-10-2011
		US 2013196324 A1	01-08-2013
		US 2013224751 A1	29-08-2013
		US 2014329239 A1	06-11-2014
		US 2016222433 A1	04-08-2016
		US 2018057863 A1	01-03-2018
		US 2022213530 A1	07-07-2022

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 24 16 1841

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11-06-2024

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		US 2023086845 A1	23-03-2023
		US 2024002910 A1	04-01-2024
		US 2024093271 A1	21-03-2024
		US 2024132939 A1	25-04-2024
		WO 2011100604 A2	18-08-2011

EPO FORM P0459

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Information on Search Strategy

The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number

EP 24 16 1841

TITLE: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

APPLICANT: California Institute of Technology

IPC CLASSIFICATION: G16B40/10, G16B25/20, C12Q1/6825, C12Q1/6851

EXAMINER: Werner, Andreas

CONSULTED DATABASES: EPODOC, BIOSIS, EMBASE, MEDLINE, NPL, WPI, XPESP, DOSYS

CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:

IPC:

CPC: G16B40/10, G16B25/20

FI/F-TERMS:

KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:
2nd-gen. divisional. System for unambiguously detecting presence/absence of each analyte of a plurality in a single volume, comprising encoding them as a 1st value of a signal (intensity). In an alternative system, the encoding scheme also uses at least one second value from a 2nd component of said signal (colour). Experimental design to increase multiplexing in a fluor. probe qPCR even when the number of channels is limited (Fig.1). Ex.1-2: For each target at least one FAM probe and 0-3 further probes with different fluorophores are combined per well (tab.15-16, Fig.2). Summing the positive control reactions provides expected cumulative signals for all possible combinations. Thus 6 different analytes are detected using only the 4 channels of the LightCycler. Ex.3: Single-color assay where 3 probes/well for 3 different targets each carry the same fluorophore but are at different concentrations, such that their respective presence can be deduced from the cumulative signal.

EPO FORM P04A42

The examination is being carried out on the **following application documents**

Description, Pages

1-73 as originally filed

Sequence listings, SEQ ID NO

1-32 received on 07-03-2024 with letter of 07-03-2024

Claims, Numbers

1-16 as originally filed

Drawings, Sheets

1/8-8/8 as originally filed

Reference is made to the following documents; the numbering will be adhered to in the rest of the procedure:

D1 HAN M ET AL: "Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules", NATURE BIOTECHNOLOGY, vol. 19, no. 7, 1 July 2001 (2001-07-01), pages 631-635, XP002545495, ISSN: 1087-0156, DOI: 10.1038/90228

D2 US 2003/148544 A1 (NIE SHUMING [US] ET AL) 7 August 2003 (2003-08-07)

D3 US 2008/124705 A1 (KRAMER FRED R [US]) 29 May 2008 (2008-05-29)

D4 XU H ET AL: "Multiplexed SNP genotyping using the Qbead system: a quantum dot-encoded microsphere-based assay", NUCLEIC ACIDS RESEARCH, vol. 31, no. 8, 15 April 2003 (2003-04-15), pages e43-1, XP002587000, ISSN: 1362-4962, DOI: 10.1093/NAR/GNG043

D5 LI Y ET AL: "Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes", NATURE BIOTECHNOLOGY, vol. 23, no. 7, 1 July 2005 (2005-07-01), pages 885-889, XP055010484, ISSN: 1087-0156, DOI: 10.1038/nbt1106 ;

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California Institute of Technology - Ex. 2026, Page 429

-& LI Y ET AL: "Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes. Supplementary Note 2", Nature Biotechnology, 1 July 2005 (2005-07-01), pages 1-1, XP093170473, Retrieved from the Internet: URL:https://static-content.springer.com/esm/art:10.1038/nbt1106/MediaObjects/41587_2005_BFnbt1106_MOESM11_ESM.pdf

D6 LIN C ET AL: "Self-Assembled Combinatorial Encoding Nanoarrays for Multiplexed Biosensing", NANO LETTERS, vol. 7, no. 2, 1 February 2007 (2007-02-01), pages 507-512, XP055031573, ISSN: 1530-6984, DOI: 10.1021/nl062998n

D7 US 2002/146734 A1 (ORTYN WILLIAM E [US] ET AL) 10 October 2002 (2002-10-10)

D8 WO 2011/100604 A2 (RAINDANCE TECHNOLOGIES INC [US]; LARSON JONATHAN WILLIAM [US] ET AL.) 18 August 2011 (2011-08-18)

1 Divisional application, amendments

1.1 The present application is a second-generation divisional derived from EP18181686.9 (granted, EP3544015B1), which is in turn derived from EP13744261.2 (granted, EP2809813B1).

1.2 The applicant provided basis for the claims in a table submitted on 06-03-2024 together with form 1001. Present claims are based on [0005], [0033]-[0034], [0055], [0063], [0084], [00107], [00118]-[00119], [00127]-[00128], [00130], [00137], [00150], [00154]-[00156], [00159], [00162], [00167] and Fig.5 of the PCT publication WO2013116780A1, corresponding to [0004], [0032]-[0033], [0053], [0061], [0082], [00105], [00116]-[00117], [00125]-[00126], [00128], [00135], [00148], [00152]-[00154], [00157], [00160], [00165] and Fig.5 of the parent divisional as filed.

1.3 Claim 2 appears to lack basis (Art. 76(1) EPC), because it omits the additional feature, present in the basis in [0034]/[0033] recited by the applicant, that *when degeneracy is eliminated, said system is capable of unambiguously detecting the presence or absence of each of at least six analytes.* This feature is presented unconditionally, and thus appears to be an essential feature of the specific system of [0034]/[0033] (GL H-V, 3.1). By contrast, "*six or more*" is merely one possible embodiment of claim 2, see dependent claim 10(a).

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1.4 It is noted that the indicated basis in [00118]-[00119]/[00116]-[00117] refers to the *methods*, *compositions* and *assays*, but not to the *systems* of the invention. Thus the new ranges in claim 10 lack basis (Art. 76(1) EPC).

1.5 Taking into account the back-references in claims 13-15 to preceding claims (e.g. the ranges in claim 10), there seems to be no direct and unambiguous basis (Art. 76(1) EPC) for the specific combinations that require selections from different lists (numbers of *analytes*, *first values* and/or *second values*). The claims single out new sub-selections.

1.6 Claim 15 specifies an *instrument configured to detect signal from six different fluorophores*, but the passage cited as basis ([00162]/[00160]) concerns compositions and kits. The other recited passage which does relate to *instruments* ([00154]/[00152]) also provides no direct and unambiguous basis (Art. 76(1) EPC). The six different fluorophores included in the composition/kit do not necessarily disclose directly and unambiguously that there is a single instrument to detect them all, see for example sequential detection of different colors discussed in D7, [0026].

1.7 Claims 11 and 16 refer to *any of the preceding claims*, and additionally specifies *droplet digital PCR*, or a *dynamic array*. However, the alleged basis [00156]/[00154] merely states that the methods of the invention are applicable to such technologies. There is lack of direct and unambiguous disclosure (Art. 76(1) EPC) that the systems according to claims 1-2, which are based on [0033]-[0034], [0084]/[0032]-[0033], [0082] would be configured for *droplet digital PCR* or a *dynamic array*.

1.8 The present description and figures comply with the requirements of Art. 76(1) EPC: They seem identical to the PCT publication of the grand-parent application EP13744261.2 (WO2013116780A1), save for the deletion of original [0001], [0043], with further paragraphs renumbered and PCT claims annexed as numbered clauses in [00187], as already presented with the parent divisional. Moreover, the application incorporates an error correction of nucleic acid sequences on pages 50-52 and correct orientation in the sequence listing (already deemed allowable for the parent application under Rule 139 EPC on the basis of [00174], which is [00172] of the present divisional).

2 Disclosure, clarity, support, conciseness (Art. 83, 84 EPC; Rule 43 EPC)

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2.1 Present claim 1 relies on system features with a given desired property or effect on the analytes, namely *generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy.*

Similarly, claim 2 requires *generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces degeneracy*.

This subject-matter is defined in terms of a "result to be achieved", without providing the skilled person with sufficient guidance how to achieve the desideratum (*performed in a manner that eliminates degeneracy*), which is unclear (Art. 84 EPC; GL F-IV, 4.10). The fact that the *first value of a signal* and/or the *second value of a signal* shall be amenable to step (d) of *cumulatively measuring* does not clarify to a sufficient degree that the values correlate to an amount, are additive, and the increments are chosen such that the sums are non-degenerate.

Corresponding objections apply to claim 8 (unambiguous detection *without immobilization, separation, mass spectrometry, or melting curve analysis*) and claim 14 (*performed in a manner that eliminates degeneracy*).

In the absence of a clear definition of the non-degenerate coding scheme that would allow to do this, this definition by a "result to be achieved" is unclear (Art. 84 EPC; GL F-IV, 4.10).

2.2 The *first value of a signal* in claims 1-2 does not comply with Art. 83 EPC (enablement) and Art. 84 EPC (clarity and support):

Art. 83 EPC not only entails that the skilled person shall be provided with at least one way of carrying out the invention (Rule 42(1)(e) EPC; GL F-III, 1), but the invention also must be enabled over essentially the whole area protected by the claims. Yet the only *first value of a signal* for which the invention has been shown to work is an *intensity or range of intensities*, and there seems to be no common general knowledge about other "values of signals" that could be used in the same way. Faced with the current, broad claim, the skilled person is confronted with undue burden, even when applying a sensible reading. Moreover, it cannot be an undue restriction to limit the claim to what has been actually invented, as opposed to staking out a claim on future inventions yet to be made.

Regarding Art. 84 EPC, support is lacking for anything else but intensity values, see e.g. [0004], [0007], and Examples. It remains unclear what other embodiments might serve as a *first value of a signal*.

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Analogous objections apply *mutatis mutandis* to the *second value* of claim 2.

The examiner notes that claim 3 would cure these issues for claim 2.

2.3 Claims 1-2 are directed to two alternative *systems.* The conditions laid down in Rule 43(2)(c) EPC appear not to be met by said claims, because they do not relate to alternative solutions to a particular problem that could not be covered by a single claim (Art. 84 EPC; GL F-IV, 3.2).

2.4 In claim 13, the feature *preferably 6* is not a proper embodiment because claim 13 requires *fewer than six* (lack of consistency and clarity, Art. 84 EPC).

3 Novelty (Art. 54(1)(2) EPC)

3.1 Having regard to claim 1, D1 discloses a *system for unambiguously detecting the presence or absence of each analyte of a plurality of analytes in a single volume, in any combination of presence or absence,*

> D1, abstract: biomolecular assay using multicolor barcoding to achieve a high degree of multiplexing, comprising creating a two-parameter optical barcode by embedding quantum dots in polystyrene microbeads as fluorophores for wavelength-and-intensity multiplexing. p.634 col.2 par.1-3, p. 635 col.2 par.3, Fig.5-6: Wavelength-resolved fluorescence spectroscopy (disclosing corresponding systems).

comprising:
(a) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy;

> D1, p.631 col.2 par.2, Fig.1A: The diagrams of Fig.1A [compare to figs. 1 and 3 of the present application] show cumulative fluorescence intensity [i.e. *cumulative measurements*] vs. wavelength for each of a number of microbeads, distinguishable according to their specific concentrations of quantum dots of different colors, which may be precisely modulated for coding. Thereby, *n* intensity levels [= *a first component of a signal*] with *m* colors generate (n^m - 1) unique codes for detecting analytes. Fig.1A, caption: Both absolute intensities and relative intensity ratios at different wavelengths are used for coding purposes; for example, (1:1:1), (2:2:2) and (2:1:1) are distinguishable codes. Fig.5: Targets #2-#4 are distinguishable by fluorescence intensity ratios 1:1:1, 1:2:1, 2:1:1.

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(b) providing a sample comprising, or potentially comprising, at least one of said analytes;

D1, p.635 col.2 par.1, "Experimental Protocol": 5'-biotinylated target oligos were first labeled with avidin-Cascade Blue; Fig.5: Targets #2-#4.

(c) contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present;

D1, p.635 col.2 par.1, "Experimental Protocol": The labeled 5'-biotinylated target oligos were then hybridized to the oligo probes in 0.1% sodium dodecyl sulfate PBS buffer at 40°C for 30 min; Fig.5: Targets #2-#4 are contacted with probes #2-#4.

(d) cumulatively measuring said first value of said signal within said sample, thereby providing a cumulative measurement;

D1, p.634 col.2 par.3, Fig.6: assay results of one mismatched and three complementary oligos hybridized to triple-color encoded beads. The code 1:1:1 corresponds to the oligo probe 5'-TCA AGG CTC AGT TCG AAT GCA CCA TA-3'. No analyte fluorescence was detected when control oligos (noncomplementary sequences) were used for hybridization (Fig. 6A). p.635 col.2 par.1, "Experimental Protocol": multiplexed assays - all targets are analyzed together [i.e. as a mixture].

and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

D1, p.634 col.2 par.3, Fig.6: assay results of one mismatched and three complementary oligos hybridized to triple-color encoded beads. The code 1:1:1 corresponds to the oligo probe 5'-TCA AGG CTC AGT TCG AAT GCA CCA TA-3'. No analyte fluorescence was detected when control oligos (noncomplementary sequences) were used for hybridization (Fig. 6A). D1 uses triple-color encoded beads (see p.634 col.2 par.1-3 and Fig.5-6).

Therefore claim 1 seems to lack novelty (Art. 54(1)(2) EPC).

Analogous objections arise in view of D2, the patent application corresponding to D1, with equivalent disclosure (D2, see passages cited in the search report).

The examiner notes that D1-D2 do not disclose cumulative measurements across all analytes in a single sample without immobilization, but rather analyze individual beads separately by immobilization (see D1, Fig.1 (caption) and Fig.5; D2, [0018]). Individual beads correspond to single targets. A cumulative fluorescence intensity level of a single bead corresponds to a cumulative signal from a

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single target. Yet this seems to be covered by the present, broad claim wording, where the entire measuring step starts out with a single sample but then involves immobilization in certain downstream steps.

3.2 Having further regard to claim 1, D3 also discloses a *system for unambiguously detecting the presence or absence of each analyte of a plurality of analytes in a single volume, in any combination of presence or absence,*

D3, [0002]-[0005]; [0008]-[0012]: multiplexed homogeneous assays for screening samples for the presence of a target nucleic acid sequence from among at least ten possible targets, utilizing conventional fluorescence detection equipment and techniques, and fluorescently labeled hybridization probes.

comprising:
(a) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy;

D3, [0011]-[0012]: Multiplex capability is achieved by combinatorial coding, including up to four different fluorophores per code element, wherein individual probes are subdivided into portions, each being labeled to emit a different color signal. [0020]; [0035]: The combinatorial coding by different colors/wavelengths is preferably balanced in intensity. [0003]; [0014]; [0032]; [0041]: However, in addition it may be supplemented with ratio coding. Taking the case where ratio coding is also used, assume one color is common to two targets, but its intensity ratio for the more abundant target is 2:1 as compared to the other portions for that target. In a real-time amplification assay three colors will rise above background as the first threshold is reached, but one color (the common color) will have a higher slope than the other two colors.

In illustrative Example 4, ten different probes are distinguishable by ten three-color codes, and the simultaneous presence of two different targets (having two colors in common but differing in the third) is deduced from the cumulative fluorescence intensity of two colors being twice as high as that of the third color (see [0048], Fig.5 and "first ternary color codebook").

(b) providing a sample comprising, or potentially comprising, at least one of said analytes;

D3, Examples 1, 4: DNA isolated from a normally sterile blood sample suspected of containing an infectious bacterium.

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(c) contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present;

D3, Example 1, [0043]; Example 4, [0048]: a real-time PCR amplification assay is carried out on. The sets of primers that are present in the reaction mixture enable the amplification of a segment of the 16S ribosomal RNA gene that is present in all of the bacterial species that are suspected of possibly being present in the blood sample. A multiplicity of molecular beacon probes is present in the assay, one for each of the suspected bacterial target species. Each different molecular beacon possesses a universal quencher moiety (dabcyl) covalently linked to its 3' end.

(d) cumulatively measuring said first value of said signal within said sample, thereby providing a cumulative measurement;

D3, Example 4, [0048], Fig.5: A signal consisting of four different colors (a, b, c, and d) arises at the same threshold cycle. The slope of the curves for two of the colors (a and b) is twice as high as the slope of the curves for the other two colors (c and d).

and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

D3, Example 4, [0048], Fig.5: This result indicates that two different bacterial species are present in the sample at approximately the same concentration, and that the three-color codes of their respective probes have two colors in common.

Therefore claim 1 lacks novelty in view of D3 (Art. 54(1)(2) EPC).

The examiner notes that D3 also discloses other Examples with ambiguous results that do not eliminate degeneracy. Example 5 (see D3, [0051], Fig.6a and 7) states that non-unique combinations may be used, and Example 7 (see D3, [0052], Fig.6b and 8) suggests to address this ambiguity by performing a second assay, wherein both the first and the second assay are degenerate. However, this cannot distract from the fact that Example 4 explicitly provides for a cumulative measurement where the results "unambiguously indicate that the sample contains species "0" and species "1" in approximately equal concentration" (Example 4, [0048], Fig.5).

3.3 D4 discloses an assay for multiplexed SNP genotyping, comprising creating unique spectral 'barcodes' combining mixtures of quantum dots with distinct emission wavelengths and intensities that enable high levels of multiplexing (abstract), such as a 10-plex PCR (p.2 col.2 par.2). The authors use nanocrystals

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of two emission colors (green, 530 and yellow, 565 nm) at concentrations to yield three intensity levels (high, medium and low), which provided for nine unique codes, plus a tenth code with no nanocrystals present (p.3 col.1 par.2). Flow cytometric analysis identifies each encoded microsphere population by its unique fluorescence profile of green and yellow light emitted at different intensity levels (Fig.2). The theoretical encoding capacity is $C = N^m-1$, wherein N is the number of intensity levels and m is the number of emission colors (p.2 col.1 par. 2). Suitable systems are provided.

Therefore claim 1 lacks novelty in view of D4 (Art. 54(1)(2) EPC).

The examiner notes that D4 does not disclose cumulative measurements across all analytes, in a single sample volume, without immobilization, but rather measures individual beads by flow cytometry (D4, abstract, Fig.2). Yet this seems to be covered by the present, broad claim wording.

3.4 D5 discloses dendrimer-like DNA-based fluorescence intensity-coded nanobarcodes that contain a probe for molecular recognition; which may be applied to multiplex detection of pathogen DNA (abstract). Following self-assembly, Fig.1 (right-hand side) shows diagrams of cumulative relative fluorescence intensity vs. wavelength. Suitable systems are provided.

Therefore claim 1 lacks novelty in view of D5 (Art. 54(1)(2) EPC).

The examiner notes that D5 does not disclose cumulative measurements across all analytes, in a single sample volume, without immobilization, but rather shows measuring five molecular probes by separate nanobarcodes containing a different fluorescence wavelength-and-intensity profile, which are individually resolved using electrophoresis (D5, Fig.1d). Yet this seems to be covered by the present, broad claim wording.

3.5 D6 discloses multiplexed detection of nucleic acids from a single solution, comprising combinatorial self-assembly of DNA nanotiles into micrometer-sized two-dimensional arrays that carry nucleic acid probes and barcoded fluorescent dyes (abstract; Fig.1). With two encoding dyes, the maximum number of barcodes of the multiplex detection system is determined by the number of different intensity levels in the two encoding channels ("red" and "green") that can be distinguished by the fluorescence microscope detector (p.508 col.1 par.1). A target molecule that binds to a specific probe displaces the probe from the array (D6, p.508 col.2 l.8). The individual target molecule then causes the disappearance of a particular colour on the tile array, so that the particular array changes to a different colour visible by a fluorescence microscope (D6, p.508 col.2 l.20). Suitable systems are provided.

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Therefore claim 1 lacks novelty in view of D6 (Art. 54(1)(2) EPC).

The examiner notes that D6 does not disclose cumulative measurements across all analytes, in a single sample volume, without immobilization, but rather measures spatially-separated arrays or "nanotiles" using a fluorescent microscope, with each nanotile configured to detect a target molecule. Spatial separation of the arrays is required (D6, p.509 col.2 l.21). Yet this seems to be covered by the present, broad claim wording.

3.6 D7 discloses labeling methods for FISH that address the need to enable analysis of a large number of cellular probes with only a limited number of fluorescent colors (D7, [0002]-[0003], last sentence). This is achieved by multiplexing multiple discriminable signaling elements per feature (or object) as a means of increasing the number of different probes that can be employed and discriminated in a cell, namely by using a binary color encoding scheme in which each feature is distinguished based on its unique color combination of signaling elements (D7, [0014]; [0059]). Using *C* colors, the number of detectable color combinations and therefore the number of uniquely identifiable features *P* in the binary encoding scheme is 2^C-1 (D7, [0015]). In the embodiment illustrated in Fig. 2C, the multiplexed signal depicts three separate features (1, 2, and 3), each associated with a different combination of intensity-coded signaling elements. Signaling elements associated with each individual feature produce a distinct image portion 444a, 444b, and 444c, wherein intensities A and B are measured cumulatively (D7, [0065], Fig.2C). Suitable systems are provided.

Therefore claim 1 lacks novelty in view of D7 (Art. 54(1)(2) EPC).

The examiner notes that D7 does not disclose cumulative measurements across all analytes, in a single sample volume, without immobilization, but rather requires spatial separation of its probes, wherein multiplexed probes within a cell are discriminated by simultaneously acquiring a set of images of the object, each image being of a different spectral band and having sufficient spatial resolution to resolve the locations of features within the object (D7, [0025]). Yet this seems to be covered by the present, broad claim wording.

3.7 The subject-matter of dependent claims 4-7, 9-10, 12 and 15 (when depending from claim 1) lacks novelty (Art. 54(1)(2) EPC), as follows:

3.7.1 Regarding claim 4, any of D2 and D3 discloses an analyte comprising *polynucleotides*, and reagents comprising *a hybrdization probe comprising a fluorophore and a quencher* (see D2, [0024], [0086]-[0090], Fig.7; D3, [0010], [0014]-[0015]).

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Moreover, D4 also indicates that its "Qbead" system can be adapted to molecular beacon probes (D4, p.7 col.2 par.4).

3.7.2 Regarding claim 5, D1 already discloses using a fluorescence microscope (Nikon Diaphot) equipped with a single-stage spectrograph (Model 270M, Spex, Edison, NJ) and a thermoelectrically cooled charge-coupled device (CCD) detector (Princeton Instruments, Trenton, NJ) [= *a photodetector*]. Near-UV excitation (330-385 nm) was provided by a 100 W mercury lamp [= *an excitation light source*]. See D1, p.634 col.2 par.1-3, p.635 col.2 par.3, Fig.5-6.

This subject-matter is also disclosed at least implicitly in D2, [0070]; and D3, [0020].

D4, D5 disclose using a flow cytometer (D4, p.3 col.2 par.3; D5, p.889 col.1 par. 3), implicitly anticipating the features of claim 5.

This also applies to D6, which discloses using a fluorescence microscope (D6, p.508 col.2 par.2), and to D7, which discloses a pixilated fluorescence detector (D7, [0028]-[0031], [0083]-[0086], [0093]-[0094], Fig.3).

3.7.3 Regarding claims 6-7, D3, [0020] discloses instruments which may either use a single-wavelength source or a multi-wavelength source, for example a white light combined with selectable filters, multiple laser sources or multiple light-emitting diode sources. D7 additionally discloses bandpass filters (D7, [0097] [0098], Fig.16-17).

3.7.4 Regarding claim 9, the *signal is generated by a fluorophore* in any of D1-D7 (D1, abstract; D2, [0016], [0018], [0069]-[0070], Fig.1; D3, [0010], D4-D7, abstracts).

3.7.5 Regarding claim 10 featuring certain ranges of numbers of analytes that may be uniquely detected, in D1 and D2, the use of 10 intensity levels (0, 1, 2,... 9) at a single wavelength (color) gives 9 unique codes, but could be expanded to give 999 codes, or 10,000-40,000 codes, or 1 million codes (D1, p.631 col.2 par.2; D2, [0046]).

In D3, combinatorial coding using colors and intensities allows to detect as many as 28 or even 56 different combinations (D3, [0013]-[0014]). D3 also discloses a kit of reagents for use in the highly multiplexed in vitro assay that can detect a minimum of six different nucleic acid targets (D3, [0019], claims 13, 14, 17).

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In D4, 10 different SNPs are distinguished (D4, Table 1), but 2-3 orders of magnitude greater levels of encoding are possible by making use of additional colors ranging from blue to far red, as well as additional intensity levels (D4, p.3 col.2 par.1).

In D5, Supplementary Note 2 (D5&) discusses theoretical and practical coding capacity, indicating that a range of up to 325 different nanobarcodes can be fabricated.

In D6, detection of four targets is exemplified (Fig.1), but indicates that, with two encoding dyes, the maximum number of barcodes of the multiplex detection system is only limited by the number of different intensity levels in the two encoding channels ("red" and "green") that can be distinguished by the fluorescence microscope detector (D6, p.508 col.1 par.1).

In D7, for example, four colors and four intensities result in 255 uniquely distinguishable combinations (D7, [0015]-[0019], Fig.1).

3.7.6 Regarding claim 12, *software* is implied by e.g. D3, [0035] or D7, [0092] disclosing a fluorescence detection instrument which can be programmed.

3.7.7 Regarding claim 15, the instruments of D1-D2 (D2, [0046], [0070]) and D3 (D3, claim 1, [0008], [0020], [0033], [0039]) appear to be configured to detect signals from six different fluorophores. Flow cytometers (as used in D4-D5) may also detect six different colors (see D7, [0005]). The pixilated detection device of D7 may also be configured for six-color detection (D7, [0101]).

3.8 Novelty is acknowledged for claims 2-3, 8, 11, 13-14 and 16.

4 Inventive step (Art. 56 EPC)

4.1 *Closest prior art and distinguishing features*

4.1.1 D1 may be considered the closest prior art.

(Though not elaborated herein, its corresponding patent application D2, or alternatively D3, appear equally suitable as closest prior art.)

4.1.2 Regarding claim 2, D1 discloses *a system for detecting the presence or absence of each analyte of a plurality of analytes in a single volume, in any combination of presence or absence,*

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D1, abstract: biomolecular assay using multicolor barcoding to achieve a high degree of multiplexing, comprising creating a two-parameter optical barcode by embedding quantum dots in polystyrene microbeads as fluorophores for wavelength-and-intensity multiplexing. p.634 col.2 par.1-3, p. 635 col.2 par.3, Fig.5-6: Wavelength-resolved fluorescence spectroscopy.

comprising:
(a) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces degeneracy;

D1, p.631 col.2 par.2, Fig.1A: The diagrams of Fig.1A [compare to figs. 1 and 3 of the present application] show cumulative fluorescence intensity [i.e. *cumulative measurements*] vs. wavelength for each of a number of microbeads, distinguishable according to their specific concentrations of quantum dots of different colors, which may be precisely modulated for coding. Thereby, *n* intensity levels [= *a first component of a signal*] with *m* colors [= *a second component of a signal*] generate (n^m - 1) unique codes for detecting analytes. Fig.1A, caption: Both absolute intensities and relative intensity ratios at different wavelengths are used for coding purposes; for example, (1:1:1), (2:2:2) and (2:1:1) are distinguishable codes. Fig.5: Targets #2-#4 are distinguishable by fluorescence intensity ratios 1:1:1, 1:2:1, 2:1:1.

(b) providing a sample comprising, or potentially comprising, at least one of said analytes;

D1, p.635 col.2 par.1, "Experimental Protocol": 5'-biotinylated target oligos were first labeled with avidin-Cascade Blue; Fig.5: Targets #2-#4.

(c) contacting said sample with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present;

D1, p.635 col.2 par.1, "Experimental Protocol": The labeled 5'-biotinylated target oligos were then hybridized to the oligo probes in 0.1% sodium dodecyl sulfate PBS buffer at 40°C for 30 min; Fig.5: Targets #2-#4 are contacted with probes #2-#4.

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(d) cumulatively measuring said first value and said second value within said sample, thereby providing a cumulative measurement;

D1, p.634 col.2 par.3, Fig.6: assay results of one mismatched and three complementary oligos hybridized to triple-color encoded beads. The code 1:1:1 corresponds to the oligo probe 5'-TCA AGG CTC AGT TCG AAT GCA CCA TA-3'. No analyte fluorescence was detected when control oligos (noncomplementary sequences) were used for hybridization (Fig. 6A). p.635 col.2 par.1, "Experimental Protocol": multiplexed assays - all targets are analyzed together [i.e. as a mixture].

and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme ~~when each of said reagents generates only one second value~~.

The distinguishing feature relates to the read-out being from only one second value, e.g. color.

By contrast, D1 uses triple-color encoded beads, see results of one mismatched and three complementary oligos hybridized to triple-color encoded beads (D1, p.634 col.2 par.1-3 and Fig.5-6). Herein, the code 1:1:1 corresponds to the oligo probe 5'-TCA AGG CTC AGT TCG AAT GCA CCA TA-3'. No analyte fluorescence was detected when control oligos (noncomplementary sequences) were used for hybridization (Fig. 6A).

Starting from D1, the problem seems to be the provision of an alternative coding scheme.

However, claim 2 fails to provide the necessary details that would allow the skilled person to solve this problem over the whole scope of the claim. The deficiencies pointed out in items 2.1 and 2.2 are not addressed. Therefore claim 2 fails to solve the problem it purports to address, and does not involve an inventive step (Art. 56 EPC).

4.2 Regarding claim 3, any of D1-D3 already discloses combining intensity and wavelength, see passages cited above. Hence this additional feature cannot contribute to inventive step (Art. 56 EPC).

4.3 Regarding claim 8, any of D1-D3 may serve as closest prior art. Claim 8 is distinguished from the above-cited prior art by negative features which set out a technical problem (how to achieve unambiguous detection of analytes *without immobilization, separation, mass spectrometry, or melting curve analysis*).

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However, claim 8 also fails to provide the necessary details that would allow the skilled person to achieve this objective over the whole scope of the claim. The deficiencies pointed out in items 2.1 and 2.2 are not addressed. Therefore claim 8 fails to solve the problem it purports to address, and does not involve an inventive step (Art. 56 EPC).

4.4 Regarding claims 13-14, specific encoding choices are within the ordinary capability of a skilled person starting from any of D1-D3, and do not seem to entail an inventive step (Art. 56 EPC).

4.5 Regarding claims 11 and 16, starting from any of D1-D3 as closest prior art, the technical effect and objective problem is the adaptation to a *droplet digital PCR system*, or *digital PCR methods to allow multiplexing in a droplet or a dynamic array*.

Document D8 already teaches such systems, including for multiplex applications (D8, see entire document). Moreover, the solution of claims 11 and 16 does not disclose any particular modifications or adaptations that would have required inventive skills. Hence a skilled person combining D1 or D3 with this D8 would have obviously arrived at the solution of claims 11 and 16 (Art. 56 EPC).

5 Further remarks

5.1 The applicant is requested to take into account the above remarks and file an amended set of claims.

5.2 Currently, no double-patenting issues arise with regard to the parent application (Art. 125 EPC, GL G-IV, 5.4 and G 4/19). Should the applicant contemplate amendments, these should be made keeping in mind the prohibition of double patenting (Art. 125 EPC; GL G-IV, 5.4 and G 4/19).

5.3 The description must be brought into conformity with the new claims to be filed; care should be taken during revision, especially of the introductory portion including any statements of problem or advantage, not to add subject-matter which extends beyond the content of the application as originally filed (Art. 123(2) EPC).

5.4 To meet the requirements of Rule 42(1)(b) EPC, at least documents D1-D3 should be identified in the description and the relevant background art disclosed therein should be briefly discussed. The two-part form is not insisted upon only

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if it is made sufficiently clear from the indication of prior art in the description which features necessary for the definition of the claimed subject-matter are, in combination, known/not known from this prior art (GL F-IV, 2.3.2).

5.5 The 74 claim-like clauses appended in [00187] at the end of the description (p. 64-73) must be deleted, since they otherwise may lead to unclarity as to the actual scope of protection (GL F-IV, 4.4).

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Applicant/Proprietor	
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Communication

The extended European search report is enclosed.

The extended European search report includes, pursuant to Rule 62 EPC, the European search report (R. 61 EPC) or the partial European search report/ declaration of no search (R. 63 EPC) and the European search opinion.

Copies of documents cited in the European search report are attached.

☒ 0 additional set(s) of copies of such documents is (are) enclosed as well.

The following have been approved:

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The following figure(s) will be published together with the abstract: none

Refund of search fee
If applicable under Article 9 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

Should you wish to further prosecute this application in the examination phase, your attention is drawn to the provisions of Rule 70a EPC. An invitation to respond to the extended European search report will be issued once the date of publication of the European search report has been mentioned in the European Patent Bulletin (R. 69(1), R. 70(2) EPC).

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EUROPEAN SEARCH REPORT

Application Number
EP 24 16 1844

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	US 2008/124705 A1 (KRAMER FRED R [US]) 29 May 2008 (2008-05-29) * paragraph [0002] - paragraph [0005] * * paragraph [0008] - paragraph [0014] * * paragraph [0016] * * paragraph [0019]; claims 13, 14, 17 * * paragraph [0032] * * paragraph [0041] * * paragraph [0043]; example 1 * * paragraph [0048]; figure 5; example 4 * * [0051]-[0052]; figures 6a, 6b, 7, 8; examples 5, 7 *	1-6	INV. G16B40/10 G16B25/20 C12Q1/6825 C12Q1/6851
A	HAN M ET AL: "Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules", NATURE BIOTECHNOLOGY, NATURE PUBLISHING GROUP, US, vol. 19, no. 7, 1 July 2001 (2001-07-01), pages 631-635, XP002545495, ISSN: 1087-0156, DOI: 10.1038/90228 * abstract * * figures 1, 5-6 * * p.631 col.2 par.2 * * p.634 col.2 par.3 * * p.635 col.2 par.1 *	1-6	TECHNICAL FIELDS SEARCHED (IPC) G16B C12Q
A	US 2003/148544 A1 (NIE SHUMING [US] ET AL) 7 August 2003 (2003-08-07) * abstract * * paragraph [0014] - paragraph [0017] * * paragraph [0018]; figure 1 * * paragraph [0045] - paragraph [0047] * * paragraph [0075] - paragraph [0079]; figure 6 *	1-6	

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	6 June 2024	Werner, Andreas

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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EUROPEAN SEARCH REPORT

Application Number
EP 24 16 1844

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A	XU H ET AL: "Multiplexed SNP genotyping using the Qbead system: a quantum dot-encoded microsphere-based assay", NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, GB, vol. 31, no. 8, 15 April 2003 (2003-04-15), pages e43-1, XP002587000, ISSN: 1362-4962, DOI: 10.1093/NAR/GNG043 * abstract * * page 2, column 1, paragraph 2 - column 2, paragraph 2 * * page 3, column 1, paragraph 2 - column 2, paragraph 1; table 1 * * figure 2 *	1-6	
A	LI Y ET AL: "Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes", NATURE BIOTECHNOLOGY, vol. 23, no. 7, 1 July 2005 (2005-07-01), pages 885-889, XP055010484, ISSN: 1087-0156, DOI: 10.1038/nbt1106 * abstract * * figure 1 * -& Li Yougen ET AL: "Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes. Supplementary Note 2.", Nature Biotechnology, 1 July 2005 (2005-07-01), pages 1-1, XP093170473, Retrieved from the Internet: URL:https://static-content.springer.com/esm/art:10.1038/nbt1106/MediaObjects/41587_2005_BFnbt1106_MOESM11_ESM.pdf [retrieved on 2024-06-04] * the whole document *	1-6	TECHNICAL FIELDS SEARCHED (IPC)

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
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EUROPEAN SEARCH REPORT

Application Number
EP 24 16 1844

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A	LIN C ET AL: "Self-Assembled Combinatorial Encoding Nanoarrays for Multiplexed Biosensing", NANO LETTERS, vol. 7, no. 2, 1 February 2007 (2007-02-01), pages 507-512, XP055031573, ISSN: 1530-6984, DOI: 10.1021/nl062998n * abstract * * figure 1 * * p.508 col.1 par.1, col.2 l.8, col.2 l.20 * * p.509 col.2 l.21 *	1-6	
A	US 2002/146734 A1 (ORTYN WILLIAM E [US] ET AL) 10 October 2002 (2002-10-10) * abstract * * paragraph [0002] - paragraph [0003] * * paragraph [0014] - paragraph [0019]; figure 1 * * paragraph [0025] * * paragraph [0059] * * paragraph [0065]; figure 2C *	1-6	
			TECHNICAL FIELDS SEARCHED (IPC)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	6 June 2024	Werner, Andreas

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ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 24 16 1844

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

06-06-2024

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2008124705 A1	29-05-2008	AU 2004236725 A1	18-11-2004
		CA 2524080 A1	18-11-2004
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		EP 1629108 A2	01-03-2006
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		JP 2007525184 A	06-09-2007
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		US 2008213792 A1	04-09-2008
		WO 2004099434 A2	18-11-2004
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		US 2002146734 A1	10-10-2002
		WO 02100157 A2	19-12-2002

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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Information on Search Strategy

The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number

EP 24 16 1844

TITLE: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

APPLICANT: California Institute of Technology

IPC CLASSIFICATION: G16B40/10, G16B25/20, C12Q1/6825, C12Q1/6851

EXAMINER: Werner, Andreas

CONSULTED DATABASES: EPODOC, BIOSIS, EMBASE, MEDLINE, NPL, WPI, XPESP, DOSYS

CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:

IPC:

CPC: G16B40/10, G16B25/20

FI/F-TERMS:

KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:
2nd-gen. divisional. Kit for unambiguously detecting the presence or absence of each of 7 analytes; comprising a coding scheme or a decoding matrix to convert a cumulative measurement to a result indicating the presence or absence of each of a plurality of analytes, wherein the probes are represented in the coding scheme. Experimental design to increase multiplexing in a fluor. probe qPCR even when the number of channels is limited (Fig.1). Ex.1-2: For each target at least one FAM probe and 0-3 further probes with different fluorophores are combined per well (tab.15-16, Fig.2). Summing the positive control reactions provides expected cumulative signals for all possible combinations. Thus 6 different analytes are detected using only the 4 channels of the LightCycler. Ex.3: Single-color assay where 3 probes/well for 3 different targets each carry the same fluorophore but are at different concentrations, such that their respective presence can be deduced from the cumulative signal.

EPO FORM P04A42

The examination is being carried out on the **following application documents**

Description, Pages

1-73 as originally filed

Sequence listings, SEQ ID NO

1-32 received on 07-03-2024 with letter of 07-03-2024

Claims, Numbers

1-6 as originally filed

Drawings, Sheets

1/8-8/8 as originally filed

Reference is made to the following documents; the numbering will be adhered to in the rest of the procedure:

D1 US 2008/124705 A1 (KRAMER FRED R [US]) 29 May 2008 (2008-05-29)

1 Divisional application, amendments

1.1 The present application is a second-generation divisional derived from EP18181686.9 (granted, EP3544015B1), which is in turn derived from EP13744261.2 (granted, EP2809813B1).

1.2 The applicant provided an indication of basis for the claims in the table filed on 06-03-2024 (together with form 1001). Accordingly, present claims are allegedly based on original claims 37, 40 and paragraphs [0023], [0027]-[0028], [0041], [0055], [0057], [00115], [00122], [00164] of PCT publication WO2013116780A1, corresponding to numbered clauses 37, 40 in [00187], and paragraphs [0022], [0026]-[0027], [0040], [0053], [0055], [00113], [00120], [00162] of the parent divisional as filed. See also Fig.6.

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1.3 Claim 1 appears to lack basis (Art. 76(1) EPC): The alleged basis for *intensity* in [0057]/[0055] is an intermediate generalisation, because said passage refers more specifically to a *fluorescence intensity.* Moreover, the two properties seem inextricably linked throughout the description (GL H-V, 3.2.1).

The objection is addressed in claim 4.

1.4 Claims 5-6 appear to lack basis (Art. 76(1) EPC), because the recited PCT claims/numbered clauses 37 and 40 are embodiments of the *method* of claim 1, rather than describing components of a *kit* (product). At best, [00164]/[00162] indicates that a kit may be *provided in suitable packaging*, may comprise *a coding scheme or a decoding matrix*, and/or *may contain any of the compositions provided in this disclosure*, but this does not read on to the features of present claims 5-6. See also ambiguity in construing the scope of these claims, as explained in item 2.3, below. A suitable basis must be direct and unambiguous.

1.5 The present description and figures comply with the requirements of Art. 76(1) EPC: They seem identical to the PCT publication of the grand-parent application EP13744261.2 (WO2013116780A1), save for the deletion of original [0001], [0043], with further paragraphs renumbered and PCT claims annexed as numbered clauses in [00187], as already presented with the parent divisional. Moreover, the application incorporates an error correction of nucleic acid sequences on pages 50-52 and correct orientation in the sequence listing (already deemed allowable for the parent application under Rule 139 EPC on the basis of [00174], which is [00172] of the present divisional).

2 Clarity (Art. 84 EPC)

2.1 Claim 1 is directed to a product (*kit*, i.e. a composition of parts) defined in part by structural features (*a bottle comprising probes for the detection of each analyte, a bottle comprising primers for amplification, a bottle comprising reagents for amplification*; and *a coding scheme or a decoding matrix*), and in part by functional features referring to an intended use.

For the functional part of the definition, it is unclear what limitations are implied by the intended use (GL F-IV, 4.13.1). This is exacerbated by the fact that the desired functions are defined *ex negativo* and in terms of a "result to be achieved" (GL F-IV, 4.10), namely being suitable *for unambiguously detecting the presence or absence of each of 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis*; and wherein the probes in the *bottle comprising probes* are chosen such that they *are represented* in a *coding*

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scheme that is a suitably designed *to convert a cumulative measurement to a result indicating the presence or absence of each of a plurality of analytes*, while also being *non-degenerate*.

The skilled person is not provided with sufficient guidance how to compose a kit that would meet these desiderata. The fact that a cumulative measurement is to be used does not clarify to a sufficient degree that the relative quantities/concentrations/amounts of the probes in the bottle must be chosen such that they yield cumulative *first values* of an *intensity or range of intensity* which *are non-degenerate.*

In the absence of a clear definition of the non-degenerate coding scheme, claim 1 is deemed unclear (Art. 84 EPC).

2.2 It is also noted that claim 1 requires that *probes are represented in the coding scheme*, but the *coding scheme* is just one of two alternatives (the other being the *decoding matrix*, linked with "*or*"). This gives rise to inconsistency with the other features that explicitly refer to the *coding scheme*, but not the second alternative.

2.3 Claims 5-6 are ambiguous and unclear (Art. 84 EPC), because *instructions [...] located on a computer in communication with a thermal cycler* might refer to executable instructions (a computer program), or alternatively to instructions directed to a human user (e.g. the PDF file of an instruction sheet).

Moreover, ambiguity arises through the reference to other entities (*computer, thermal cycler*) that are not part of the claimed first entity (*kit*) but related to it through use (see GL F-IV, 4.14 and 4.14.1). Are they part of the kit or not?

3 Novelty (Art. 54(1)(2) EPC)

3.1 Novelty is acknowledged for claims 1-6.

4 Inventive step (Art. 56 EPC)

4.1 *Assessment of inventive step of mixed-type inventions*

The present claims comprise technical and non-technical features. The assessment of inventive step is therefore carried out in accordance with G-VII, 5.4 of the Guidelines for Examination.

In the case of claims comprising technical and non-technical features, only those features which contribute to the technical character of the invention are taken into account for the assessment of inventive step.

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4.2 *Closest prior art and distinguishing features*

4.2.1 D1 is taken as the closest prior art.

4.2.2 D1 discloses a *kit for unambiguously detecting the presence or absence of each of 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis, comprising:*

~~*a bottle comprising*~~ *probes for the detection of each analyte,*

~~*a bottle comprising*~~ *primers for amplification, and*

~~*a bottle comprising*~~ *reagents for amplification;*

D1 discloses kits comprising quenched fluorescence detection probes [i.e. *hybridisation probes*] and primers for amplification. See also D1, [0019] and claims 13, 14 and 17, which discloses a

> kit of reagents for use in a highly multiplexed in vitro assay to screen a sample for the presence of any of a minimum of six different nucleic acid targets comprising a set of fluorescent hybridization probes including at least six combinatorially coded fluorescent hybridization probes, each specific for a different nucleic acid target, whose hybridization to their targets can be detected in said assay by a fluorescent signal change indicative of their labeling, wherein the probes in the set are combinatorially coded with up to four different emitter fluorophores from a panel of at least four spectrally differentiable fluorophores by labeling portions of each probe with different emitter fluorophores.

See also D1, Example 1, [0043]; Example 4, [0048]: a real-time PCR amplification assay is carried out on. The sets of primers that are present in the reaction mixture enable the amplification of a segment of the 16S ribosomal RNA gene that is present in all of the bacterial species that are suspected of possibly being present in the blood sample. A multiplicity of molecular beacon probes is present in the assay, one for each of the suspected bacterial target species. Each different molecular beacon possesses a universal quencher moiety (dabcyl) covalently linked to its 3' end.

Note: Since D1 describes "six" as the minimum number of a range, detection of 7 analytes is also implicitly anticipated. D1 also teaches that combinatorial coding using colors and intensities allows to detect as many as 28 or even 56 different combinations (D1, [0013]-[0014]).

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~~*a coding scheme or a decoding matrix to convert a cumulative measurement to a result indicating the presence or absence of each of a plurality of analytes, wherein the probes are represented in the coding scheme*~~

D1, [0011]-[0012]: Multiplex capability is achieved by combinatorial coding, including up to four different fluorophores per code element, wherein individual probes are subdivided into portions, each being labeled to emit a different color signal. [0020]; [0035]: The combinatorial coding by different colors/wavelengths is preferably balanced in intensity.

In illustrative Example 4, ten different probes are distinguishable by ten three-color codes, and the simultaneous presence of two different targets (having two colors in common but differing in the third) is deduced from the cumulative fluorescence intensity of two colors being twice as high as that of the third color (see [0048], Fig.5 and "first ternary color codebook").

However, D1 does not disclose packaging *a coding scheme or a decoding matrix* together with the kit reagents.

wherein, the cumulative measurement is generated by measuring a first value of a signal within the sample,

D1, Example 4, [0048], Fig.5: A signal consisting of four different colors (a, b, c, and d) arises at the same threshold cycle. The slope of the curves for two of the colors (a and b) is twice as high as the slope of the curves for the other two colors (c and d).

the first value is an intensity or range of intensities,

D1, [0003], [0014], [0032], [0041]: Taking the case where ratio coding is also used, assume one color is common to two targets, but its intensity ratio for the more abundant target is 2:1 as compared to the other portions for that target. In a real-time amplification assay three colors will rise above background as the first threshold is reached, but one color (the common color) will have a higher slope than the other two colors.

the coding scheme is non-degenerate, and

D1, Example 4 explicitly provides for a cumulative measurement where the results "unambiguously indicate that the sample contains species "0" and species "1" in approximately equal concentration" (Example 4, [0048], Fig. 5).

the analyte is a polynucleotide analyte.

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D1, e.g. Example 4: DNA isolated from a normally sterile blood sample suspected of containing an infectious bacterium. A real-time PCR amplification assay is carried out on. The sets of primers that are present in the reaction mixture enable the amplification of a segment of the 16S ribosomal RNA gene that is present in all of the bacterial species that are suspected of possibly being present in the blood sample.

4.3 *Analysis of the technical character of the distinguishing features*

4.3.1 The distinguishing features - when taken in isolation - are in part non-technical because the *coding scheme* or *decoding matrix* is interpreted as a "presentation of information" in the sense of Art. 52(2)(d) and (3) EPC (GL G-II, 3.7).

Features which are non-technical when taken in isolation may nevertheless contribute to the technical character of an invention if, in the context of the invention, they contribute to producing a technical effect serving a technical purpose (GL G-VII, 5.4, second paragraph).

The subject-matter in the present case is characterized by the cognitive content of the information (the "what") to be processed by the mind of the user (as opposed to functional data to be processed by a technical system). A feature defining a "presentation of information" may produce a technical effect if it credibly assists the user in performing a technical task by means of a continued and/or guided human-machine interaction process. As indicated in GL G-II, 3.7, such a technical effect might arise if the cognitive content of the information presented to the user relates to an internal state prevailing in a technical system and would enable the user to properly operate this technical system. However, static or predetermined information about operating instructions does not qualify as an internal state prevailing in the device. This is the situation for the claimed data structures.

Hence the presentations of information covered by the *coding scheme* or *decoding matrix* cannot contribute to inventive step.

4.3.2 The remaining part of the distinguishing features identified above (the *bottles*) contributes to the technical character of the invention.

4.4 *Problem and solution*

The only remaining distinguishing features are the *bottles* which are used to package the *primers*, *probes* and *reagents*.

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The problem, and its trivial solution, would have been considered by a skilled person starting from D1 without exercising inventive skills (Art. 56 EPC), taking into account common general knowledge.

4.5 The additional features of dependent claims 3-4 (*polymerase chain reaction, fluorescent signal*) are already known from D1 (see D1, [0019], claims 13, 14, 17, Example 1, [0043]; Example 4, [0048]).

4.6 Dependent claims 2 and 5-6 provide further non-technical features; however, these fail to address the fundamental lack of contribution to technical character argued above. It is noted that contribution to technical character must be present for essentially all embodiments falling within the ambit of a claim.

With more particular regard to the *instruction* embodiments of claims 5-6, irrespective of storage on a computer, the claim encompasses static or predetermined information about operating instructions directed to cognitive processing by a human user. The cognitive content is not limited to dynamically changing information in relation to an internal state prevailing in a technical system that would enable the user to properly operate this technical system. Thus assistance to a user in performing a technical task through a continued and/or guided human-machine interaction process is not credibly achieved, at least not over the whole scope of the claim (see objections in items 2.3 and 4.3.1, above). It is noted that a technical effect is considered credibly achieved if the assistance to the user in performing the technical task is objectively, reliably and causally linked to the feature (GL G-II, 3.7). No suitable fall-back position is apparent either.

5 Further remarks

5.1 At least some of the objections raised above are such that there appears to be no possibility of overcoming them by amendment. Refusal of the application under Art. 97(2) EPC is therefore to be expected.

5.2 Should the applicant nevertheless contemplate filing amended claims, an independent claim should be filed taking account of Rule 43(1) EPC, indicating the basis for the amendments.

Non-obviousness of the claimed subject-matter has to be demonstrated by defining the difference between the claimed subject-matter and the teaching of the closest prior art.

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For any non-technical distinguishing features, it will be necessary to substantiate why said features are considered by the applicant to make a technical contribution over D1, i.e. to contribute to a technical effect for solving a technical problem over D1.

According to this difference the technical problem underlying the alleged invention has to be defined and the proposed solution (i.e. the subject-matter of the next version of the claims) has to be demonstrated to be non-obviously derivable from the closest prior art. This should be shown e.g. by indicating advantageous and unexpected technical properties or effects of said next version of the claimed subject-matter.

5.3 Any amendments to this second-generation divisional should be made keeping in mind the prohibition of double patenting (Art. 125 EPC; GL G-IV, 5.4 and G 4/19).

5.4 To meet the requirements of Rule 42(1)(b) EPC, document D1 should be identified in the description and the relevant background art disclosed therein should be briefly discussed. The two-part form is not insisted upon only if it is made sufficiently clear from the indication of prior art in the description which features necessary for the definition of the claimed subject-matter are, in combination, known/not known from this prior art (GL F-IV, 2.3.2).

5.5 The 74 claim-like clauses appended in [00187] at the end of the description (p. 64-73) must be deleted, since they otherwise may lead to unclarity as to the actual scope of protection (GL F-IV, 4.4).

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Date 18.06.2024

Reference	Application No./Patent No.
P087509EP	24161820.6 - 1122 / 4361607
Applicant/Proprietor: California Institute of Technology	

Communication

The extended European search report is enclosed.

The extended European search report includes, pursuant to Rule 62 EPC, the European search report (R. 61 EPC) or the partial European search report/ declaration of no search (R. 63 EPC) and the European search opinion.

Copies of documents cited in the European search report are attached.

☒ 0 additional set(s) of copies of such documents is (are) enclosed as well.

The following have been approved:

☒ Abstract ☒ Title

☐ The Abstract was modified and the definitive text is attached to this communication.

The following figure(s) will be published together with the abstract: none

Refund of search fee
If applicable under Article 9 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

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EUROPEAN SEARCH REPORT

Application Number
EP 24 16 1820

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A	HAN M ET AL: "Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules", NATURE BIOTECHNOLOGY, NATURE PUBLISHING GROUP, US, vol. 19, no. 7, 1 July 2001 (2001-07-01), pages 631-635, XP002545495, ISSN: 1087-0156, DOI: 10.1038/90228 * abstract * * figures 1, 5-6 * * p.631 col.2 par.2 * * p.634 col.2 par.3 * * p.635 col.2 par.1 *	1-13	INV. G16B40/10 G16B25/20 C12Q1/6825 C12Q1/6851
A	US 2003/148544 A1 (NIE SHUMING [US] ET AL) 7 August 2003 (2003-08-07) * abstract * * paragraph [0014] - paragraph [0017] * * paragraph [0018]; figure 1 * * paragraph [0045] - paragraph [0047] * * paragraph [0075] - paragraph [0079]; figure 6 *	1-13	TECHNICAL FIELDS SEARCHED (IPC) G16B C12Q
A	US 2008/124705 A1 (KRAMER FRED R [US]) 29 May 2008 (2008-05-29) * paragraph [0002] - paragraph [0005] * * paragraph [0008] - paragraph [0014] * * paragraph [0016] * * paragraph [0019]; claims 13, 14, 17 * * paragraph [0032] * * paragraph [0041] * * paragraph [0043]; example 1 * * paragraph [0048]; figure 5; example 4 * * [0051]-[0052]; figures 6a, 6b, 7, 8; examples 5, 7 *	1-13	

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	5 June 2024	Werner, Andreas

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

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Europäisches Patentamt
European Patent Office
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EUROPEAN SEARCH REPORT

Application Number
EP 24 16 1820

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A	XU H ET AL: "Multiplexed SNP genotyping using the Qbead system: a quantum dot-encoded microsphere-based assay", NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, GB, vol. 31, no. 8, 15 April 2003 (2003-04-15), pages e43-1, XP002587000, ISSN: 1362-4962, DOI: 10.1093/NAR/GNG043 * abstract * * page 2, column 1, paragraph 2 - column 2, paragraph 2 * * page 3, column 1, paragraph 2 - column 2, paragraph 1; table 1 * * figure 2 * -----	1-13	
A	LI Y ET AL: "Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes", NATURE BIOTECHNOLOGY, vol. 23, no. 7, 1 July 2005 (2005-07-01), pages 885-889, XP055010484, ISSN: 1087-0156, DOI: 10.1038/nbt1106 * abstract * * figure 1 * -& Li Yougen ET AL: "Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes. Supplementary Note 2.", Nature Biotechnology, 1 July 2005 (2005-07-01), pages 1-1, XP093170473, Retrieved from the Internet: URL:https://static-content.springer.com/esm/art:10.1038/nbt1106/MediaObjects/41587_2005_BFnbt1106_MOESM11_ESM.pdf [retrieved on 2024-06-04] ----- -/--	1-13	TECHNICAL FIELDS SEARCHED (IPC)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	5 June 2024	Werner, Andreas

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EUROPEAN SEARCH REPORT

Application Number
EP 24 16 1820

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A	LIN C ET AL: "Self-Assembled Combinatorial Encoding Nanoarrays for Multiplexed Biosensing", NANO LETTERS, vol. 7, no. 2, 1 February 2007 (2007-02-01), pages 507-512, XP055031573, ISSN: 1530-6984, DOI: 10.1021/nl062998n * abstract * * figure 1 * * p.508 col.1 par.1, col.2 l.8, col.2 l.20 * * p.509 col.2 l.21 *	1-13	
A	US 2002/146734 A1 (ORTYN WILLIAM E [US] ET AL) 10 October 2002 (2002-10-10) * abstract * * paragraph [0002] - paragraph [0003] * * paragraph [0014] - paragraph [0019]; figure 1 * * paragraph [0059] * * paragraph [0065]; figure 2C * * paragraph [0025] *	1-13	
			TECHNICAL FIELDS SEARCHED (IPC)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	5 June 2024	Werner, Andreas

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ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO.

EP 24 16 1820

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

05-06-2024

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2003148544 A1	07-08-2003	AU 2002322348 A1	03-03-2003
		CA 2450725 A1	09-01-2003
		EP 1410031 A2	21-04-2004
		JP 2005508493 A	31-03-2005
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		US 2002146734 A1	10-10-2002
		WO 02100157 A2	19-12-2002

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

Information on Search Strategy

The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number

EP 24 16 1820

TITLE: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

APPLICANT: California Institute of Technology

IPC CLASSIFICATION: G16B40/10, G16B25/20, C12Q1/6825, C12Q1/6851

EXAMINER: Werner, Andreas

CONSULTED DATABASES: EPODOC, BIOSIS, EMBASE, MEDLINE, NPL, WPI, XPESP, DOSYS

CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:

IPC:

CPC: G16B40/10, G16B25/20

FI/F-TERMS:

KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:
2nd-gen. divisional. Method of unambiguously detecting the presence or absence of each analyte of a plurality of analytes in a single reaction mixture, comprising encoding each analyte as at least one intensity of a fluorescence emission signal, thereby generating a nondegenerate coding scheme for the plurality of M analytes. Experimental design to increase multiplexing in a fluor. probe qPCR even when the number of channels is limited (Fig.1). Ex.1-2: For each target at least one FAM probe and 0-3 further probes with different fluorophores are combined per well (tab.15-16, Fig.2). Summing the positive control reactions provides expected cumulative signals for all possible combinations. Thus 6 different analytes are detected using only the 4 channels of the LightCycler. Ex.3: Single-color assay where 3 probes/well for 3 different targets each carry the same fluorophore but are at different concentrations, such that their respective presence can be deduced from the cumulative signal.

EPO FORM P04A42

The examination is being carried out on the **following application documents**

Description, Pages

1-73	as originally filed			

Sequence listings, SEQ ID NO

1-32	received on	07-03-2024	with letter of	07-03-2024

Claims, Numbers

1-13	as originally filed

Drawings, Sheets

1/8-8/8	as originally filed

Reference is made to the following documents; the numbering will be adhered to in the rest of the procedure:

D1 HAN M ET AL: "Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules", NATURE BIOTECHNOLOGY, vol. 19, no. 7, 1 July 2001 (2001-07-01), pages 631-635, XP002545495, ISSN: 1087-0156, DOI: 10.1038/90228

D2 US 2003/148544 A1 (NIE SHUMING [US] ET AL) 7 August 2003 (2003-08-07)

D3 US 2008/124705 A1 (KRAMER FRED R [US]) 29 May 2008 (2008-05-29)

D4 XU H ET AL: "Multiplexed SNP genotyping using the Qbead system: a quantum dot-encoded microsphere-based assay", NUCLEIC ACIDS RESEARCH, vol. 31, no. 8, 15 April 2003 (2003-04-15), pages e43-1, XP002587000, ISSN: 1362-4962, DOI: 10.1093/NAR/GNG043

D5 LI Y ET AL: "Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes", NATURE BIOTECHNOLOGY, vol. 23, no. 7, 1 July 2005 (2005-07-01), pages 885-889, XP055010484, ISSN: 1087-0156, DOI: 10.1038/nbt1106 ;

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-& LI Y ET AL: "Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes. Supplementary Note 2", Nature Biotechnology, 1 July 2005 (2005-07-01), pages 1-1, XP093170473, Retrieved from the Internet: URL:https://static-content.springer.com/esm/art:10.1038/nbt1106/MediaObjects/41587_2005_BFnbt1106_MOESM11_ESM.pdf

D6 LIN C ET AL: "Self-Assembled Combinatorial Encoding Nanoarrays for Multiplexed Biosensing", NANO LETTERS, vol. 7, no. 2, 1 February 2007 (2007-02-01), pages 507-512, XP055031573, ISSN: 1530-6984, DOI: 10.1021/nl062998n

D7 US 2002/146734 A1 (ORTYN WILLIAM E [US] ET AL) 10 October 2002 (2002-10-10)

1 Divisional application, amendments

1.1 The present application is a second-generation divisional derived from EP18181686.9 (granted, EP3544015B1), which is in turn derived from EP13744261.2 (granted, EP2809813B1).

1.2 The basis for the claims provided by the applicant on 06-03-2024 together with form 1001 has been checked and appears to be correct. Present claims are based on [0005], [0010], [0027]-[0028], [0055], [00114], [00119], [00121] of the PCT publication WO2013116780A1, corresponding to [0004], [0009], [0026]-[0027], [0053], [00114], [00117], [00119] of the parent divisional as filed.

1.3 The present description and figures are identical to that of PCT publication WO2013116780A1, save for the deletion of original [0001], [0043], and renumbered paragraphs, as already presented with the parent divisional, and annexing of PCT claims as numbered clauses in [00187].

Moreover, the application incorporates an error correction of nucleic acid sequences on pages 50-52 and correct orientation in the sequence listing (already deemed allowable for the parent application under Rule 139 EPC on the basis of [00174], which is [00172] of the present divisional).

1.4 Therefore the application seems to comply with the requirements of Art. 76(1) EPC.

2 Novelty (Art. 54(1)(2) EPC)

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2.1 Novelty is acknowledged for claims 1-13, because none of D1-D7 discloses all features of claim 1.

3 Inventive step (Art. 56 EPC)

3.1 *Closest prior art and distinguishing features*

3.1.1 D1 may be considered the closest prior art. (D2, the corresponding patent application, would be a suitable alternative closest prior art.)

3.1.2 D1 discloses a *method of unambiguously detecting the presence or absence of each analyte of a plurality of M analytes ~~in a single reaction mixture~~, comprising*

> D1, abstract: biomolecular assay using multicolor barcoding to achieve a high degree of multiplexing, comprising creating a two-parameter optical barcode by embedding quantum dots in polystyrene microbeads as fluorophores for wavelength-and-intensity multiplexing.

encoding each analyte as at least one intensity (I) of a fluorescence emission signal, thereby generating a nondegenerate coding scheme for the plurality of M analytes,

> D1, p.631 col.2 par.2, Fig.1A: The diagrams of Fig.1A [compare to figs. 1 and 3 of the present application] show cumulative fluorescence intensity [i.e. *cumulative measurements*] vs. wavelength for each of a number of microbeads, distinguishable according to their specific concentrations of quantum dots of different colors, which may be precisely modulated for coding. Thereby, *n* intensity levels with *m* colors generate (n^m - 1) unique codes for detecting analytes. Fig.1A, caption: Both absolute intensities and relative intensity ratios at different wavelengths are used for coding purposes; for example, (1:1:1), (2:2:2) and (2:1:1) are distinguishable codes. Fig.5: Targets #2-#4 are distinguishable by fluorescence intensity ratios 1:1:1, 1:2:1, 2:1:1.

providing a sample comprising, or potentially comprising, at least one of said analytes;

> D1, p.635 col.2 par.1, "Experimental Protocol": 5'-biotinylated target oligos were first labeled with avidin-Cascade Blue; Fig.5: Targets #2-#4.

contacting the sample with a plurality of analyte-specific reagents to generate a fluorescence emission intensity (I),

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D1, p.635 col.2 par.1, "Experimental Protocol": The labeled 5'-biotinylated target oligos were then hybridized to the oligo probes in 0.1% sodium dodecyl sulfate PBS buffer at 40°C for 30 min; Fig.5: Targets #2-#4 are contacted with probes #2-#4. Multiplexed assays - all targets are analyzed together [i.e. as a mixture].

cumulatively measuring the (i) ~~generated from the single reaction mixture~~, thereby providing a cumulative intensity measurement, and

D1, p.634 col.2 par.3, Fig.6: assay results of one mismatched and three complementary oligos hybridized to triple-color encoded beads. The code 1:1:1 corresponds to the oligo probe 5'-TCA AGG CTC AGT TCG AAT GCA CCA TA-3'. No analyte fluorescence was detected when control oligos (noncomplementary sequences) were used for hybridization (Fig. 6A). p.635 col.2 par.1, "Experimental Protocol":

determining for each analyte ~~in the single reaction mixture~~, whether the analyte is present or absent based on the cumulative intensity measurement and the (I) in the coding scheme.

D1, p.634 col.2 par.3, Fig.6: assay results of one mismatched and three complementary oligos hybridized to triple-color encoded beads. The code 1:1:1 corresponds to the oligo probe 5'-TCA AGG CTC AGT TCG AAT GCA CCA TA-3'. No analyte fluorescence was detected when control oligos (noncomplementary sequences) were used for hybridization (Fig. 6A).

The distinguishing features are highlighted in ~~*strikethrough*~~ font.

3.2 The technical effect of the distinguishing features is that multiplexing is achieved by a single cumulative measurement across analytes *in a single reaction mixture*. In D1-D2, individual beads must be analyzed separately (D1, legend of Fig.1, p.632, Fig.5; D2, [0018]).

The technical problem to be solved by the application may be considered the provision of a simplified multiplexed assay.

3.3 The solution is not obvious over the prior art:

- In D1-D2, individual beads correspond to single targets, and a cumulative fluorescence intensity level of a single bead corresponds to a cumulative signal from a single target. D1-D2 analyze individual beads separately by immobilization, which requires separately measuring individual beads corresponding to single targets (see D1, Fig.1 (caption) and Fig.5; D2, [0018]), not a single cumulative measurement across a plurality of analytes *in a single reaction mixture*, i.e. in bulk solution, which is much simpler to implement. Starting from D1-D2, a

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skilled person had no pointer that the coding scheme of claim 1 could be applied without immobilization *in a single reaction mixture*, unless resorting to unallowable hindsight.

The absence of any pointer to a cumulative measurement across analytes in a single reaction mixture cannot be compensated by the teachings of any of D3-D7:

- D3 discloses a distinct scheme (see Examples 5 and 7 of D3, namely [0051]) that resolves ambiguity by performing a second (or further) assay(s), each of which is degenerate. For example, the "second ternary color codebook" in the table shown besides [0053] discloses a code of "abcdde" that can indicate either the probes for species 0 ("cde") and 8 ("abd") or the probes for species 1 ("bde") and species 6 ("acd").

- D4 uses flow cytometry to measure individual microspheres, wherein each SNP is distinguished by its unique spectral barcode (abstract; Fig.2).

- D5 individually measures each of five molecular probes that are separately encoded by nano-barcodes with different fluorescence wavelength-and-intensity profiles (see exemplary embodiment shown in Fig.1).

- D6 measures spatially-separated arrays or "nanotiles" using a fluorescent microscope (p.508 col.2 l.8), which requires spatial separation of the arrays and spacing between the tiles to prevent energy transfer processes (p.509 col.2 l. 21).

- D7 analyzes corresponding locations across an image set to determine the spectral composition and therefore the probe identity at each location in the object ([0025]). In exemplary Fig. 2C of D7, three separate features (1, 2, and 3) are each associated with a different combination of intensity-coded signaling elements, and signaling elements associated with each individual feature produce a distinct image portion 444a, 444b, and 444c ([0065]).

Thus D1-D2 alone or in combination with D3-D7 fail to suggest an assay as defined in present claim 1. The method of claim 1 is therefore inventive (Art. 56 EPC).

3.4 Further claims 2-13 are inventive, because they share the inventive features of said independent claim 1.

4 Further remarks

4.1 Once the the applicant has taken into account the remarks below, a communication under Rule 71(3) EPC could be readily issued.

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4.2 Currently, no double-patenting issues arise with regard to the parent application (Art. 125 EPC, GL G-IV, 5.4 and G 4/19). The examiner notes similarity of claim 1 to claim 1 of EP13744261.2 as granted. Should the applicant contemplate amendments, these should be made keeping in mind the prohibition of double patenting (Art. 125 EPC; GL G-IV, 5.4 and G 4/19).

4.3 The description must be brought into conformity with the new claims to be filed; care should be taken during revision, especially of the introductory portion including any statements of problem or advantage, not to add subject-matter which extends beyond the content of the application as originally filed (Art. 123(2) EPC).

4.4 To meet the requirements of Rule 42(1)(b) EPC, at least documents D1-D3 should be identified in the description and the relevant background art disclosed therein should be briefly discussed. The two-part form is not insisted upon only if it is made sufficiently clear from the indication of prior art in the description which features necessary for the definition of the claimed subject-matter are, in combination, known/not known from this prior art (GL F-IV, 2.3.2).

4.5 The 74 claim-like clauses appended in [00187] at the end of the description (p. 64-73) must be deleted, since they otherwise may lead to unclarity as to the actual scope of protection (GL F-IV, 4.4).

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APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/352,112	07/24/2024 05:00:39 PM Z ET	88DG-373535-US

Title of Invention

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	3145	FILED BY	Annie Wong
PATENT CENTER #	66507118	AUTHORIZED BY	David Heisey
CUSTOMER #	30764	FILING DATE	07/13/2023
CORRESPONDENCE ADDRESS	-	FIRST NAMED INVENTOR	Emil P. KARTALOV

Payment Information

PAYMENT METHOD	PAYMENT TRANSACTION ID	PAYMENT AUTHORIZED BY
DA / 504561	E20247NH05446513	Annie Wong

FEE CODE	DESCRIPTION	ITEM PRICE($)	QUANTITY	ITEM TOTAL($)
2806	SUBMISSION OF AN INFORMATION DISCLOSURE STATEMENT	104.00	1	104.00
			TOTAL AMOUNT:	$104.00

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 filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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

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

New International Application Filed with the USPTO as a Receiving Office

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

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Date 12.06.2024

Reference	Application No./Patent No.
P087510EP	24161817.2 - 1122 / 4361606
Applicant/Proprietor	
California Institute of Technology	

Communication

The extended European search report is enclosed.

The extended European search report includes, pursuant to Rule 62 EPC, the European search report (R. 61 EPC) or the partial European search report/ declaration of no search (R. 63 EPC) and the European search opinion.

Copies of documents cited in the European search report are attached.

☒ 0 additional set(s) of copies of such documents is (are) enclosed as well.

The following have been approved:

☒ Abstract ☒ Title

☐ The Abstract was modified and the definitive text is attached to this communication.

The following figure(s) will be published together with the abstract: none

Refund of search fee
If applicable under Article 9 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

Should you wish to further prosecute this application in the examination phase, your attention is drawn to the provisions of Rule 70a EPC. An invitation to respond to the extended European search report will be issued once the date of publication of the European search report has been mentioned in the European Patent Bulletin (R. 69(1), R. 70(2) EPC).

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European Patent Office
Office européen des brevets

EUROPEAN SEARCH REPORT

Application Number
EP 24 16 1817

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-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	5 June 2024	Werner, Andreas

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E : earlier patent document, but published on, or after the filing date
D : document cited in the application
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EPO FORM 1503 03.82 (P04C01)

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EUROPEAN SEARCH REPORT

Application Number
EP 24 16 1817

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	XU H ET AL: "Multiplexed SNP genotyping using the Qbead system: a quantum dot-encoded microsphere-based assay", NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, GB, vol. 31, no. 8, 15 April 2003 (2003-04-15), pages e43-1, XP002587000, ISSN: 1362-4962, DOI: 10.1093/NAR/GNG043 * abstract * * page 2, column 1, paragraph 2 - column 2, paragraph 2 * * page 3, column 1, paragraph 2 - column 2, paragraph 1; table 1 * * figure 2 * -----	1-15	
X	LI Y ET AL: "Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes", NATURE BIOTECHNOLOGY, vol. 23, no. 7, 1 July 2005 (2005-07-01), pages 885-889, XP055010484, ISSN: 1087-0156, DOI: 10.1038/nbt1106 * abstract * * figure 1 * -& LI Y ET AL: "Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes. Supplementary Note 2.", Nature Biotechnology, 1 July 2005 (2005-07-01), pages 1-1, XP093170473, Retrieved from the Internet: URL:https://static-content.springer.com/esm/art:10.1038/nbt1106/MediaObjects/41587_2005_BFnbt1106_MOESM11_ESM.pdf [retrieved on 2024-06-04] * the whole document * ----- -/--	1-15	TECHNICAL FIELDS SEARCHED (IPC)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	5 June 2024	Werner, Andreas

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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X	LIN C ET AL: "Self-Assembled Combinatorial Encoding Nanoarrays for Multiplexed Biosensing", NANO LETTERS, vol. 7, no. 2, 1 February 2007 (2007-02-01), pages 507-512, XP055031573, ISSN: 1530-6984, DOI: 10.1021/nl062998n * abstract * * figure 1 * * p.508 col.1 par.1, col.2 l.8, col.2 l.20 * * p.509 col.2 l.21 *	1-15	
X	US 2002/146734 A1 (ORTYN WILLIAM E [US] ET AL) 10 October 2002 (2002-10-10) * abstract * * paragraph [0002] - paragraph [0003] * * paragraph [0014] - paragraph [0019]; figure 1 * * paragraph [0025] * * paragraph [0059] * * paragraph [0065]; figure 2C *	1-15	TECHNICAL FIELDS SEARCHED (IPC)
E	WO 2013/096851 A1 (HARVARD COLLEGE [US]) 27 June 2013 (2013-06-27) * [0005]-[0006], [0008]-[0010], [0014], [0039], [00129]; figure 1 *	1,3	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	5 June 2024	Werner, Andreas

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ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 24 16 1817

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
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05-06-2024

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		WO 2013096851 A1	27-06-2013

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

Information on Search Strategy
The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number
EP 24 16 1817

TITLE: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

APPLICANT: California Institute of Technology

IPC CLASSIFICATION: G16B25/20, G16B40/10, C12Q1/6825, C12Q1/6851

EXAMINER: Werner, Andreas

CONSULTED DATABASES: EPODOC, BIOSIS, EMBASE, MEDLINE, NPL, WPI, XPESP, DOSYS

CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:

IPC:

CPC: G16B40/10, G16B25/20

FI/F-TERMS:

KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:
2nd-gen. divisional. Method of detecting the presence/absence of each one of a plurality of analytes, comprising generating a coding scheme, wherein each of said analytes is represented by a first value of a signal eliminating degeneracy; comprising cumulatively measuring said first value of said signal within a sample. Experimental design to increase multiplexing in a fluor. probe qPCR even when the number of channels is limited (Fig.1). Ex.1-2: For each target at least one FAM probe and 0-3 further probes with different fluorophores are combined per well (tab.15-16, Fig.2). Summing the positive control reactions provides expected cumulative signals for all possible combinations. Thus 6 different analytes are detected using only the 4 channels of the LightCycler. Ex.3: Single-color assay where 3 probes/well for 3 different targets each carry the same fluorophore but are at different concentrations, such that their respective presence can be deduced from the cumulative signal.

EPO FORM P04A42

The examination is being carried out on the **following application documents**

Description, Pages

1-73 as originally filed

Sequence listings, SEQ ID NO

1-32 received on 07-03-2024 with letter of 07-03-2024

Claims, Numbers

1-15 as originally filed

Drawings, Sheets

1/8-8/8 as originally filed

Reference is made to the following documents; the numbering will be adhered to in the rest of the procedure:

D1 HAN M ET AL: "Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules", NATURE BIOTECHNOLOGY, vol. 19, no. 7, 1 July 2001 (2001-07-01), pages 631-635, XP002545495, ISSN: 1087-0156, DOI: 10.1038/90228

D2 US 2003/148544 A1 (NIE SHUMING [US] ET AL) 7 August 2003 (2003-08-07)

D3 US 2008/124705 A1 (KRAMER FRED R [US]) 29 May 2008 (2008-05-29)

D4 XU H ET AL: "Multiplexed SNP genotyping using the Qbead system: a quantum dot-encoded microsphere-based assay", NUCLEIC ACIDS RESEARCH, vol. 31, no. 8, 15 April 2003 (2003-04-15), pages e43-1, XP002587000, ISSN: 1362-4962, DOI: 10.1093/NAR/GNG043

D5 LI Y ET AL: "Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes", NATURE BIOTECHNOLOGY, vol. 23, no. 7, 1 July 2005 (2005-07-01), pages 885-889, XP055010484, ISSN: 1087-0156, DOI: 10.1038/nbt1106 ;

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-& LI Y ET AL: "Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes. Supplementary Note 2", Nature Biotechnology, 1 July 2005 (2005-07-01), pages 1-1, XP093170473, Retrieved from the Internet: URL:https://static-content.springer.com/esm/art:10.1038/nbt1106/MediaObjects/41587_2005_BFnbt1106_MOESM11_ESM.pdf

D6 LIN C ET AL: "Self-Assembled Combinatorial Encoding Nanoarrays for Multiplexed Biosensing", NANO LETTERS, vol. 7, no. 2, 1 February 2007 (2007-02-01), pages 507-512, XP055031573, ISSN: 1530-6984, DOI: 10.1021/nl062998n

D7 US 2002/146734 A1 (ORTYN WILLIAM E [US] ET AL) 10 October 2002 (2002-10-10)

1 Divisional application, amendments

1.1 The present application is a second-generation divisional derived from EP18181686.9 (granted, EP3544015B1), which is in turn derived from EP13744261.2 (granted, EP2809813B1).

1.2 The basis for the claims provided by the applicant on 06-03-2024 together with form 1001 has been checked and appears to be correct.

1.3 The present description and figures seem identical to that of parent application EP13744261.2 as originally filed (published as WO2013116780A1), save for the deletion of original [0001] and [0043], and annexing of PCT claims as numbered clauses in [00187].

Moreover, it incorporates an error correction of nucleic acid sequences on pages 50-52 and correct orientation in the sequence listing (already deemed allowable for the parent application under Rule 139 EPC on the basis of [00174], which is [00172] of the present divisional).

1.4 Therefore the application seems to comply with the requirements of Art. 76(1) EPC.

2 Disclosure, clarity, support (Art. 83, 84 EPC)

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2.1 Present claim 1 relates to a method which has a given desired property or effect, namely *generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy.*

This subject-matter is defined in terms of a "result to be achieved", without providing the skilled person with sufficient guidance how to achieve the desideratum (*performed in a manner that eliminates degeneracy*), which is unclear (Art. 84 EPC; GL F-IV, 4.10). The fact that the *first value of a signal* shall be amenable to step (d) of *cumulatively measuring* does not clarify to a sufficient degree that the values correlate to an *amount*, are additive, and the increments are chosen such that there is no degeneracy.

The same objection applies to claim 7, which indicates that detection would be possible *without immobilization, separation, mass spectrometry, or melting curve analysis.* In the absence of a clear definition of the non-degenerate coding scheme that would allow to do this, this definition by a "result to be achieved" is unclear (Art. 84 EPC; GL F-IV, 4.10).

The examiner is of the opinion that claims 5 or 6 could be used to cure these issues.

2.2 The *first value of a signal* in claim 1 does not comply with Art. 83 EPC (enablement) and Art. 84 EPC (clarity and support):

Art. 83 EPC not only entails that the skilled person shall be provided with at least one way of carrying out the invention (Rule 42(1)(e) EPC; GL F-III, 1), but the invention also must be enabled over essentially the whole area protected by the claims. Yet the only *first value of a signal* for which the invention has been shown to work is an *intensity or range of intensities*, and there seems to be no common general knowledge about other "values of signals" that could be used in the same way. Faced with the current, broad claim, the skilled person is confronted with undue burden, even when applying a sensible reading. Moreover, it cannot be an undue restriction to limit the claim to what has been actually invented, as opposed to staking out a claim on future inventions yet to be made.

Regarding Art. 84 EPC, support is lacking for anything else but intensity values, see e.g. [0004], [0007], and Examples. Moreover, it remains unclear what other embodiments might serve as a *first value of a signal.*

The examiner notes that claim 3 would cure these issues.

3 Novelty (Art. 54(1)(2) EPC)

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3.1 D1 discloses a *method of detecting the presence or absence of each analyte of a plurality of analytes,*

D1, abstract: biomolecular assay using multicolor barcoding to achieve a high degree of multiplexing, comprising creating a two-parameter optical barcode by embedding quantum dots in polystyrene microbeads as fluorophores for wavelength-and-intensity multiplexing.

comprising:

a. encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy;

D1, p.631 col.2 par.2, Fig.1A: The diagrams of Fig.1A [compare to figs. 1 and 3 of the present application] show cumulative fluorescence intensity [i.e. *cumulative measurements*] vs. wavelength for each of a number of microbeads, distinguishable according to their specific concentrations of quantum dots of different colors, which may be precisely modulated for coding. Thereby, n intensity levels with m colors generate $(n^m - 1)$ unique codes for detecting analytes. Fig.1A, caption: Both absolute intensities and relative intensity ratios at different wavelengths are used for coding purposes; for example, (1:1:1), (2:2:2) and (2:1:1) are distinguishable codes. Fig.5: Targets #2-#4 are distinguishable by fluorescence intensity ratios 1:1:1, 1:2:1, 2:1:1.

b. providing a sample comprising, or potentially comprising, at least one of said analytes;

D1, p.635 col.2 par.1, "Experimental Protocol": 5'-biotinylated target oligos were first labeled with avidin-Cascade Blue; Fig.5: Targets #2-#4.

c. contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present;

D1, p.635 col.2 par.1, "Experimental Protocol": The labeled 5'-biotinylated target oligos were then hybridized to the oligo probes in 0.1% sodium dodecyl sulfate PBS buffer at 40°C for 30 min; Fig.5: Targets #2-#4 are contacted with probes #2-#4.

d. cumulatively measuring said first value of said signal within said sample, thereby providing a cumulative measurement;

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D1, p.634 col.2 par.3, Fig.6: assay results of one mismatched and three complementary oligos hybridized to triple-color encoded beads. The code 1:1:1 corresponds to the oligo probe 5'-TCA AGG CTC AGT TCG AAT GCA CCA TA-3'. No analyte fluorescence was detected when control oligos (noncomplementary sequences) were used for hybridization (Fig. 6A). p.635 col.2 par.1, "Experimental Protocol": multiplexed assays - all targets are analyzed together [i.e. as a mixture].

and

e. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

D1, p.634 col.2 par.3, Fig.6: assay results of one mismatched and three complementary oligos hybridized to triple-color encoded beads. The code 1:1:1 corresponds to the oligo probe 5'-TCA AGG CTC AGT TCG AAT GCA CCA TA-3'. No analyte fluorescence was detected when control oligos (noncomplementary sequences) were used for hybridization (Fig. 6A).

D2 is the patent application corresponding to D1, with equivalent disclosure (D2, see passages cited in the search report).

Therefore claim 1 lacks novelty in view of each of D1 and D2 (Art. 54(1)(2) EPC).

The examiner notes that D1-D2 do not disclose cumulative measurements across all analytes in a single sample, without immobilization, but rather analyze individual beads separately by immobilization (see D1, Fig.1 (caption) and Fig.5; D2, [0018]). Individual beads correspond to single targets. A cumulative fluorescence intensity level of a single bead corresponds to a cumulative signal from a single target. Yet this seems to be covered by the present, broad claim wording.

3.2 D3 discloses a *method of detecting the presence or absence of each analyte of a plurality of analytes,*

D3, [0002]-[0005]; [0008]-[0012]: multiplexed homogeneous assays for screening samples for the presence of a target nucleic acid sequence from among at least ten possible targets, utilizing conventional fluorescence detection equipment and techniques, and fluorescently labeled hybridization probes.

comprising:

a. encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy;

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D3, [0011]-[0012]: Multiplex capability is achieved by combinatorial coding, including up to four different fluorophores per code element, wherein individual probes are subdivided into portions, each being labeled to emit a different color signal. [0020]; [0035]: The combinatorial coding by different colors/wavelengths is preferably balanced in intensity. [0003]; [0014]; [0032]; [0041]: However, in addition it may be supplemented with ratio coding. Taking the case where ratio coding is also used, assume one color is common to two targets, but its intensity ratio for the more abundant target is 2:1 as compared to the other portions for that target. In a real-time amplification assay three colors will rise above background as the first threshold is reached, but one color (the common color) will have a higher slope than the other two colors.

In illustrative Example 4, ten different probes are distinguishable by ten three-color codes, and the simultaneous presence of two different targets (having two colors in common but differing in the third) is deduced from the cumulative fluorescence intensity of two colors being twice as high as that of the third color (see [0048], Fig.5 and "first ternary color codebook").

b. providing a sample comprising, or potentially comprising, at least one of said analytes;

D3, Examples 1, 4: DNA isolated from a normally sterile blood sample suspected of containing an infectious bacterium.

c. contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present;

D3, Example 1, [0043]; Example 4, [0048]: a real-time PCR amplification assay is carried out on. The sets of primers that are present in the reaction mixture enable the amplification of a segment of the 16S ribosomal RNA gene that is present in all of the bacterial species that are suspected of possibly being present in the blood sample. A multiplicity of molecular beacon probes is present in the assay, one for each of the suspected bacterial target species. Each different molecular beacon possesses a universal quencher moiety (dabcyl) covalently linked to its 3' end.

d. cumulatively measuring said first value of said signal within said sample, thereby providing a cumulative measurement;

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D3, Example 4, [0048], Fig.5: A signal consisting of four different colors (a, b, c, and d) arises at the same threshold cycle. The slope of the curves for two of the colors (a and b) is twice as high as the slope of the curves for the other two colors (c and d).

and

e. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

D3, Example 4, [0048], Fig.5: This result indicates that two different bacterial species are present in the sample at approximately the same concentration, and that the three-color codes of their respective probes have two colors in common.

Therefore claim 1 lacks novelty in view of D3 (Art. 54(1)(2) EPC).

The examiner notes that D3 also discloses other Examples with ambiguous results that do not eliminate degeneracy. Example 5 (see D3, [0051], Fig.6a and 7) states that non-unique combinations may be used, and Example 7 (see D3, [0052], Fig.6b and 8) suggests to address this ambiguity by performing a second assay, wherein both the first and the second assay are degenerate. However, this cannot distract from the fact that Example 4 explicitly provides for a cumulative measurement where the results "unambiguously indicate that the sample contains species "0" and species "1" in approximately equal concentration" (Example 4, [0048], Fig.5).

3.3 D4 discloses an assay for multiplexed SNP genotyping, comprising creating unique spectral 'barcodes' combining mixtures of quantum dots with distinct emission wavelengths and intensities that enable high levels of multiplexing (abstract), such as a 10-plex PCR (p.2 col.2 par.2). The authors use nanocrystals of two emission colors (green, 530 and yellow, 565 nm) at concentrations to yield three intensity levels (high, medium and low), which provided for nine unique codes, plus a tenth code with no nanocrystals present (p.3 col.1 par.2). Flow cytometric analysis identifies each encoded microsphere population by its unique fluorescence profile of green and yellow light emitted at different intensity levels (Fig.2). The theoretical encoding capacity is $C = N^m-1$, wherein N is the number of intensity levels and m is the number of emission colors (p.2 col.1 par. 2).

Therefore claim 1 lacks novelty in view of D4 (Art. 54(1)(2) EPC).

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The examiner notes that D4 does not disclose cumulative measurements across all analytes, in a single sample volume, without immobilization, but rather measures individual beads by flow cytometry (D4, abstract, Fig.2). Yet this seems to be covered by the present, broad claim wording.

3.4 D5 discloses dendrimer-like DNA-based fluorescence intensity-coded nanobarcodes that contain a probe for molecular recognition; which may be applied to multiplex detection of pathogen DNA (abstract). Following self-assembly, Fig.1 (right-hand side) shows diagrams of cumulative relative fluorescence intensity vs. wavelength.

Therefore claim 1 lacks novelty in view of D5 (Art. 54(1)(2) EPC).

The examiner notes that D5 does not disclose cumulative measurements across all analytes, in a single sample volume, without immobilization, but rather shows measuring five molecular probes by separate nanobarcodes containing a different fluorescence wavelength-and-intensity profile, which are individually resolved using electrophoresis (D5, Fig.1d). Yet this seems to be covered by the present, broad claim wording.

3.5 D6 discloses multiplexed detection of nucleic acids from a single solution, comprising combinatorial self-assembly of DNA nanotiles into micrometer-sized two-dimensional arrays that carry nucleic acid probes and barcoded fluorescent dyes (abstract; Fig.1). With two encoding dyes, the maximum number of barcodes of the multiplex detection system is determined by the number of different intensity levels in the two encoding channels ("red" and "green") that can be distinguished by the fluorescence microscope detector (p.508 col.1 par.1). A target molecule that binds to a specific probe displaces the probe from the array (D6, p.508 col.2 l.8). The individual target molecule then causes the disappearance of a particular colour on the tile array, so that the particular array changes to a different colour visible by a fluorescence microscope (D6, p.508 col.2 l.20).

Therefore claim 1 lacks novelty in view of D6 (Art. 54(1)(2) EPC).

The examiner notes that D6 does not disclose cumulative measurements across all analytes, in a single sample volume, without immobilization, but rather measures spatially-separated arrays or "nanotiles" using a fluorescent microscope, with each nanotile configured to detect a target molecule. Spatial separation of the arrays is required (D6, p.509 col.2 l.21). Yet this seems to be covered by the present, broad claim wording.

3.6 D7 discloses labeling methods for FISH that address the need to enable analysis of a large number of cellular probes with only a limited number of fluorescent colors (D7, [0002]-[0003], last sentence). This is achieved by multiplexing multi-

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ple discriminable signaling elements per feature (or object) as a means of increasing the number of different probes that can be employed and discriminated in a cell, namely by using a binary color encoding scheme in which each feature is distinguished based on its unique color combination of signaling elements (D7, [0014]; [0059]). Using *C* colors, the number of detectable color combinations and therefore the number of uniquely identifiable features *P* in the binary encoding scheme is 2^C-1 (D7, [0015]). In the embodiment illustrated in Fig. 2C, the multiplexed signal depicts three separate features (1, 2, and 3), each associated with a different combination of intensity-coded signaling elements. Signaling elements associated with each individual feature produce a distinct image portion 444a, 444b, and 444c, wherein intensities A and B are measured cumulatively (D7, [0065], Fig.2C).

Therefore claim 1 lacks novelty in view of D7 (Art. 54(1)(2) EPC).

The examiner notes that D7 does not disclose cumulative measurements across all analytes, in a single sample volume, without immobilization, but rather requires spatial separation of its probes, wherein multiplexed probes within a cell are discriminated by simultaneously acquiring a set of images of the object, each image being of a different spectral band and having sufficient spatial resolution to resolve the locations of features within the object (D7, [0025]). Yet this seems to be covered by the present, broad claim wording.

3.7 Dependent claim 3 specifies the first value to be an *intensity or range of intensities*. This is anticipated by D1-D7 (see above).

3.8 Dependent claims 8-15 concern particular numbers of *M* ranging from four to twelve. The prior art assays described above already allow to detect this number of analytes.

For example, in D1 and D2, the use of 10 intensity levels (0, 1, 2,... 9) at a single wavelength (color) gives 9 unique codes, but could be expanded to give 999 codes, or 10,000-40,000 codes, or 1 million codes (D1, p.631 col.2 par.2; D2, [0046]).

In D3, combinatorial coding using colors and intensities allows to detect as many as 28 or even 56 different combinations (D3, [0013]-[0014]). D3 also discloses a kit of reagents for use in the highly multiplexed *in vitro* assay that can detect a minimum of six different nucleic acid targets (D3, [0019], claims 13, 14, 17).

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In D4, 10 different SNPs are distinguished (D4, Table 1), but 2-3 orders of magnitude greater levels of encoding are possible by making use of additional colors ranging from blue to far red, as well as additional intensity levels (D4, p.3 col.2 par.1).

In D5, Supplementary Note 2 (D5&) discusses theoretical and practical coding capacity, indicating that a range of up to 325 different nanobarcodes can be fabricated.

In D6, detection of four targets is exemplified (Fig.1), but indicates that, with two encoding dyes, the maximum number of barcodes of the multiplex detection system is only limited by the number of different intensity levels in the two encoding channels ("red" and "green") that can be distinguished by the fluorescence microscope detector (D6, p.508 col.1 par.1).

In D7, for example, four colors and four intensities result in 255 uniquely distinguishable combinations (D7, [0015]-[0019], Fig.1).

3.9 Novelty is acknowledged for claims 2 and 4-7.

4 Intermediate documents (Art. 54(3) EPC)

4.1 Document WO2013096851A1, cited as "E" and published on 27-06-2013, claims the priority date of 22-12-2011. It has been supplied to the European Patent Office in one of its official languages according to Art. 153(3) and (4) EPC and the filing fee provided for in Rule 159(1)(c) EPC or Art. 39(1) PCT has been paid. The requirements of Rule 165 EPC are thus fulfilled. Its content as filed is therefore considered to be comprised in the state of the art relevant to the question of novelty, pursuant to Art. 54(3) EPC.

4.2 This earlier application is from the same technical area as the present application and addresses the same problem (see [0005]-[0006]). It provides multiplex assays for detecting a plurality of analytes in a sample, by using detection reagents in soluble phase such as antibodies and barcode oligos bound to a particle; wherein the barcode oligos (comprising one or more subunits) are decoded by sequential decoding using labelled decoding probes ([0008]-[0010]; Fig.1). The methods do not require immobilization ([0039]). For example, three image sets and four optical labels allow enconding $4^3 = 64$ distinct probe reagents. In some embodiments, the relative intensity of the signal signatures can be used in identification of each subpopulation of the detection reagents. Thus, the intensity of the signal signatures can be used as part of a coding

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scheme of the detection reagents ([0014]). For example, if one detectable label is allowed to light up twice as brightly in a different color than another, the multiplexing capacity can be even further expanded ([00129]).

This document appears to be prejudicial to the novelty of the subject-matter of claims 1 and 3 (Art. 54(3) EPC).

5 Inventive step (Art. 56 EPC)

5.1 *Assessment of inventive step of mixed-type inventions*

The present claims comprise technical and non-technical features. The assessment of inventive step is therefore carried out in accordance with G-VII, 5.4 of the Guidelines for Examination.

In the case of claims comprising technical and non-technical features, only those features which contribute to the technical character of the invention are taken into account for the assessment of inventive step.

5.2 *Closest prior art and distinguishing features*

5.2.1 D1 may be considered the closest prior art. (D2, the corresponding patent application, would be a suitable alternative closest prior art.)

5.2.2 Dependent claims 2 and 4-7 differ by details on the non-degenerate coding scheme:

Claim 2 concerns a mathematical formula to determine the number *M* of analytes that can be detected as a function of the *maximum cumulative value F*.

Claim 4 concerns a list of various *minimum values* for the *first value of a signal*, ranging from *at least 1* to *at least 64*. It can be garnered from [00101]-[00102] that this refers to multiples of a discretized amount of a *first value of a signal*, which is doubled in each increment.

Claims 5-6 specify an increment *equal to*, or *greater than* the *cumulative maximum* of the *preceding first value plus one.*

Claim 7 indicates that the decoding scheme would allow unambiguous detection *without immobilization, separation, mass spectrometry, or melting curve analysis*.

5.3 *Analysis of the technical character of the distinguishing features*

5.3.1 The distinguishing features - when taken in isolation - are non-technical because they concern the abstract coding scheme, which relates to mathematics and mental acts (Art. 52(2)(a), (c) and (3) EPC).

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5.3.2 Features which are non-technical when taken in isolation may nevertheless contribute to the technical character of an invention if, in the context of the invention, they contribute to producing a technical effect serving a technical purpose (GL G-VII, 5.4, second paragraph).

The purpose of these distinguishing features is thought to be the provision of a simplified multiplexed assay. This purpose is technical.

5.3.3 However, the claim is not sufficiently limited to ensure that the distinguishing features actually produce a technical effect serving this technical purpose over substantially the whole claim scope. Reference is made to the clarity objections raised in items 2.1 and 2.2, above. Consequently, the claim fails to establish a sufficient link between the technical purpose identified above and the mathematical/algorithmic processing steps defined by the distinguishing features (GL G-II, 3.3). The claim is thus not functionally limited to the technical purpose identified above, and a technical effect is not credibly achieved.

It follows that, in the context of the present claim 1, the distinguishing features, which are non-technical in isolation, do not actually produce a technical effect serving the technical purpose identified above.

5.4 *Conclusion regarding inventive step*

As claims 2 and 4-7 do not comprise any feature making a technical contribution over the teaching of D1, they cannot be regarded as involving an inventive step within the meaning of Art. 56 EPC (GL G-VII, 5.4(iii)(b)).

5.5 *Further suggestions*

The limitation of the claimed subject-matter to a scope for which a technical effect may be acknowledged could be achieved by combining the features of claim 1, claim 3, and claims 5 or 6.

In this case, a non-obvious contribution over the prior art could be acknowledged.

6 Further remarks

6.1 The applicant is requested to take into account the above remarks and file an amended set of claims.

6.2 The examiner notes similarity of claim 1 to claim 1 of EP13744261.2 as granted. Any amendments should be made keeping in mind the prohibition of double patenting (Art. 125 EPC; GL G-IV, 5.4 and G 4/19).

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6.3 The description must be brought into conformity with the new claims to be filed; care should be taken during revision, especially of the introductory portion including any statements of problem or advantage, not to add subject-matter which extends beyond the content of the application as originally filed (Art. 123(2) EPC).

6.4 To meet the requirements of Rule 42(1)(b) EPC, at least documents D1-D3 should be identified in the description and the relevant background art disclosed therein should be briefly discussed. The two-part form is not insisted upon only if it is made sufficiently clear from the indication of prior art in the description which features necessary for the definition of the claimed subject-matter are, in combination, known/not known from this prior art (GL F-IV, 2.3.2).

6.5 The 74 claim-like clauses appended in [00187] at the end of the description (p. 64-73) must be deleted, since they otherwise may lead to unclarity as to the actual scope of protection (GL F-IV, 4.4).

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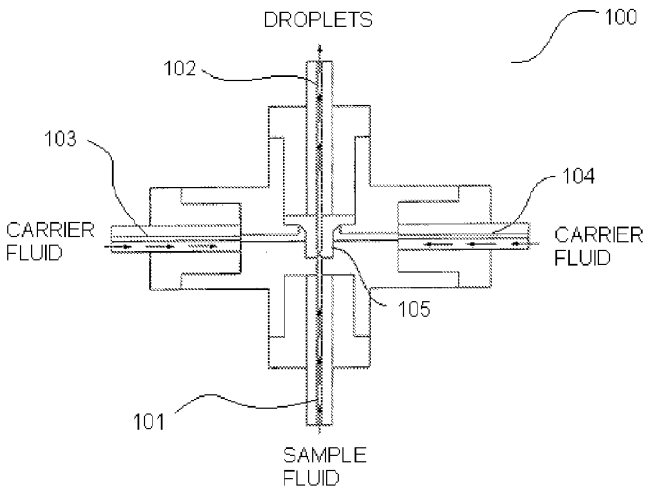
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[Continued on next page]

(54) **Title**: DIGITAL ANALYTE ANALYSIS

FIGURE 1



(57) **Abstract**: The invention generally relates to droplet based digital PCR and methods for analyzing a target nucleic acid using the same. In certain embodiments, methods of the invention involve forming sample droplets containing, on average, a single target nucleic acid, amplifying the target in the droplets, excluding droplets containing amplicon from the target and amplicon from a variant of the target, and analyzing target amplicons.

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DIGITAL ANALYTE ANALYSIS

Reference To Related Applications

This non-provisional application claims priority to U.S. Provisional Application No. 61/388,937, filed October 1, 2010, U.S. Provisional Application No. 61/347,158, filed May 21, 2010, U.S. Provisional Application No. 61/331,490, filed, May 5, 2010., and U.S. Provisional Application No. 61/304,163, filed February 12, 2010, the contents of which are each incorporated by reference herein in their entireties.

Field of the Invention

The invention generally relates to droplet based digital PCR and methods for analyzing a target nucleic acid using the same.

Background

Assays have been developed that rely on analyzing nucleic acid molecules from bodily fluids for the presence of mutations, thus leading to early diagnosis of certain diseases such as cancer. In a typical bodily fluid sample however, any abnormal nucleic acids containing mutations of interest are often present in small amounts (e.g., less than 1%) relative to a total amount of nucleic acid in the bodily fluid sample. This can result in a failure to detect the small amount of abnormal nucleic acid due to stochastic sampling bias.

The advent of PCR and real-time PCR methodologies has greatly improved the analysis of nucleic acids from both throughput and quantitative perspectives. While traditional PCR techniques typically rely on end-point, and sometimes semi-quantitative, analysis of amplified DNA targets via agarose gel electrophoresis, real-time PCR (or qPCR) methods are geared toward accurately quantifying exponential amplification as the reaction progresses. qPCR reactions are monitored either using a variety of highly sequence specific fluorescent probe technologies, or by using non-specific DNA intercalating fluorogenic dyes.

As the need for higher throughput in analyzing multiple targets in parallel continues to escalate in the fields of genomics and genetics, and as the need for more efficient use of sample grows in medically related fields such as diagnostics, the ability to perform and quantify multiple amplifications simultaneously within the same reaction volume (multiplexing) is paramount for

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both PCR and qPCR. While end-point PCR can support a high level of amplicon multiplexing, such ample capacity for multiplexing probe-based qPCR reactions remains elusive for a number of reasons. For example, most commercial real-time thermal cyclers only support up to four differently colored fluorophores for detection as a consequence of the limited spectral resolution of common fluorophores, translating into a multiplexing capacity of 4×. Additionally, while optimization of single target primer/probe reactions is now standard practice, combining primers and probes for multiple reactions changes the thermodynamic efficiencies and/or chemical kinetics, necessitating potentially extensive troubleshooting and optimization. Very high multiplexing of greater than100× has been demonstrated in a "one of many" detection format for pathogen identification using "sloppy" molecular beacons and melting points as fingerprints, however the approach is restricted to applications with a slim likelihood of the presence of multiple simultaneous targets. A half-multiplexing method achieved 19× in a two step reaction with general multiplexed preamplification in the first step, followed by separate single-plex quantitative PCR in the second step. However a general purpose single-pot solution to qPCR multiplexing does not yet exist.

Digital PCR (dPCR) is an alternative quantitation method in which dilute samples are divided into many separate reactions. See for example, Brown et al. (U.S. patent numbers 6,143,496 and 6,391,559) and Vogelstein et al. (U.S. patent numbers 6,440,706, 6,753,147, and 7,824,889), the content of each of which is incorporated by reference herein in its entirety. The distribution from background of target DNA molecules among the reactions follows Poisson statistics, and at so called "terminal dilution" the vast majority of reactions contain either one or zero target DNA molecules for practical intents and purposes. In another case, at so called "limiting dilution" some reactions contain zero DNA molecules, some reactions contain one molecule, and frequently some other reactions contain multiple molecules, following the Poisson distribution. It is understood that terminal dilution and limiting dilution are useful concepts for describing DNA loading in reaction vessels, but they have no formal mathematical definition, nor are they necessarily mutually exclusive. Ideally, at terminal dilution, the number of PCR positive reactions (PCR(+)) equals the number of template molecules originally present. At limiting dilution, Poisson statistics are used to uncover the underlying amount of DNA. The principle advantage of digital compared to qPCR is that it avoids any need to interpret the time

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dependence of fluorescence intensity—an analog signal—along with the main underlying uncertainty of non-exponential amplification during early cycles.

Summary

The invention generally relates to the manipulation of nucleic acid in droplets, and in particular, nucleic acid amplification and detection. In one aspect, the invention provides a droplet that contains a single nucleic acid template and a plurality of primer pairs specific for multiple target sites on the template. The single nucleic acid template can be DNA (e.g., genomic DNA, cDNA, etc.) or RNA. The template is amplified in the droplet for detection; and may preferably be amplified using a plurality of primer pairs as described herein.

The ability to amplify and detect single nucleic acids in droplets enables digital PCR, detection, counting, and differentiation among nucleic acids, especially those present in heterogeneous samples. Thus, the invention applies to digital amplification techniques and, in specific embodiments enables multiplex PCR in droplets. For example, multiplexing primers in droplets enables the simultaneous increase in the number of PCR droplets while keeping the amount of input DNA the same or lower and generate the same or greater amplicon yield. This results in an overall increase in the amount of PCR positive droplets and amplicon yield without the consumption of more DNA. Even though the number of PCR primer pairs per droplet is greater than one, there is only one template molecule per droplet, and thus, in some implementations, there is only one primer pair per droplet that is being utilized at one time. As such, the advantages of droplet PCR for eliminating bias from either allele specific PCR or competition between different amplicons is maintained. However, as described below in relation to detection of haplotypes, other implementations advantageously allow detection of multiple loci on a single template using multiple primer pairs, preferably designed to minimize bias.

Microfluidic droplets for multiplex analysis according to the invention contain a plurality of probes that hybridize to amplicons produced in the droplets. Preferably, the droplet contains two or more probes, e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 60, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 500, or more probes. Certain members of the plurality of probes include a detectable label. Members of the plurality of probes can each include the same detectable label, or a different detectable label. The detectable label is preferably a fluorescent label. The plurality of probes can include one or

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more groups of probes at varying concentrations. The one or more groups of probes can include the same detectable label which will vary in intensity upon detection, due to the varying probe concentrations. The droplets of the invention can further contain one or more reagents for conducting a polymerase chain reaction, such as a DNA or RNA polymerase, and/or dNTPs.

The present invention additionally relates to a method for detecting a plurality of targets in a biological sample using digital PCR in microfluidic droplets. The sample may be a human tissue or body fluid. Exemplary body fluids pus, sputum, semen, urine, blood, saliva, and cerebrospinal fluid.

One or more droplets are formed, each containing a single nucleic acid template and a heterogeneous mixture of primer pairs and probes, each specific for multiple target sites on the template. For example, a first fluid (either continuous, or discontinuous as in droplets) containing a single nucleic acid template (DNA or RNA) is merged with a second fluid (also either continuous, or discontinuous as in droplets) containing a plurality of primer pairs and a plurality of probes, each specific for multiple targets sites on the nucleic acid template to form a droplet containing the single nucleic acid template and a heterogeneous mixture of primer pairs and probes. The second fluid can also contain reagents for conducting a PCR reaction, such as a polymerase and dNTPs.

Certain members of the plurality of probes include a detectable label. Members of the plurality of probes can each include the same detectable label, or a different detectable label. The detectable label is preferably a fluorescent label. The plurality of probes can include one or more groups of probes at varying concentrations. The one or more groups of probes can include the same detectable label which varies in intensity upon detection, due to the varying probe concentrations.

The first and second fluids can each be in droplet form. Any technique known in the art for forming droplets may be used with methods of the invention. An exemplary method involves flowing a stream of the sample fluid containing the nucleic acid template such that it intersects two opposing streams of flowing carrier fluid. The carrier fluid is immiscible with the sample fluid. Intersection of the sample fluid with the two opposing streams of flowing carrier fluid results in partitioning of the sample fluid into individual sample droplets containing the first fluid. The carrier fluid may be any fluid that is immiscible with the sample fluid. An exemplary carrier fluid is oil. In certain embodiments, the carrier fluid includes a surfactant, such as a

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fluorosurfactant. The same method may be applied to create individual droplets from the second fluid containing the primer pairs (and, in some implementations, the amplification reagents). Either the droplets containing the first fluid, the droplets containing the second fluid, or both, may be formed and then stored in a library for later merging, aspects of certain implementations of which are described in U.S. Patent Application Serial No. 12/504,764, hereby incorporated herein in its entirety for all purposes. Once formed, droplets containing the first and second fluids can be merged to form single droplets containing the single nucleic acid template and heterogeneous mixture of primer pairs and probes. Merging can be accomplished, for example, in the presence of an electric field. Moreover, it is not required that both fluids be in the form of droplets when merging takes places. One exemplary method for merging of fluid portions with droplets is taught, for example, in co-pending U.S. Patent Application No. 61/441,985, filed on even date herewith.

The nucleic acid template in each of the merged/formed droplets is amplified, e.g., by thermocycling the droplets under temperatures/conditions sufficient to conduct a PCR reaction. The resulting amplicons in the droplets can then be analyzed. For example, the presence of absence of the plurality of targets in the one or more droplets is detected optically, e.g., by the detectable label on the plurality of probes.

The invention further relates to methods for analyzing a target nucleic acid. More particularly, methods of the invention are able to detect polymerase errors that occur during a PCR reaction and are able to exclude from analysis amplification products that are a result of a polymerase error. Methods of the invention are particularly useful in digital PCR where a polymerase error may result in a partitioned section of sample being incorrectly identified as containing a mutant allele, i.e., a false positive. Such false positives greatly impact the validity and precision of digital PCR results. Methods of the invention are able to uniquely detect multiple targets with the same optical color. Methods of the invention are particularly useful in digital PCR where it is desirable to identify multiple different target molecules that may be present in the starting test fluid.

Methods of the invention involve forming sample droplets containing target nucleic acid. Ideally, methods of the invention comprise forming droplets for digital PCR. Preferred digital PCR droplets contain one copy of a nucleic acid to be amplified, although they may contain multiple copies of the same nucleic acid sequence. Any technique known in the art for forming

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sample droplets may be used with methods of the invention. One exemplary method involves flowing a stream of sample fluid including nucleic acids such that it intersects two opposing streams of flowing carrier fluid. The carrier fluid is immiscible with the sample fluid. Intersection of the sample fluid with the two opposing streams of flowing carrier fluid results in partitioning of the sample fluid into individual sample droplets. The carrier fluid may be any fluid that is immiscible with the sample fluid. An exemplary carrier fluid is oil. In certain embodiments, the carrier fluid includes a surfactant, such as a fluorosurfactant.

The targets are then amplified in the droplets. Any method known in the art may be used to amplify the target nucleic acids either linearly or exponentially. A preferred method is the polymerase chain reaction (PCR). For purposes of the invention, any amplification technique commonly known in the art may be implemented such as rolling circle amplification, isothermal amplification, or any combination of amplification methods using loci specific primers, nested-primers, or random primers (such primers, and/or primers used for PCR, are included in the term "amplification reagents"). Once amplified, droplets containing amplicon from the target and amplicon from a variant of the target are excluded. One method to exclude droplets that contain a heterogeneous population of amplicons from droplets that contain a homogeneous population of amplicons includes hybridizing detectably-labeled probes to the amplicons, flowing the droplets through a microfluidic channel, and excluding those droplets in which both amplicon from the target and amplicon from a variant of the target are detected.

Once droplets containing a heterogeneous population of amplicons are excluded, droplets that contain a homogeneous population of amplicons are analyzed. Any analytical technique known in the art may be used. In certain embodiments, analyzing the droplets involves determining a number of droplets that contain only wild-type target, and determining a number of droplets that contain only a variant of the target. Generally, the presence of droplets containing only the variant is indicative of a disease, such as cancer. The variant may be an allelic variant. An exemplary allelic variant is a single nucleotide polymorphism. The variant may also be a specific haplotype. Haplotypes refer to the presence of two or more variants on the same nucleic acid strand. Haplotypes can be more informative or predictive than genotypes when used to determine such things as the presence or severity of disease, response to drug therapy or drug resistance of bacterial or viral infections. Because each droplet contains only one template strand it is an ideal vessel for the determination of haplotypes. The detection of

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two or more variants in a single droplet that contains a single intact nucleic acid strand identifies the haplotype of the variants on that strand. The presence of two or more markers in the same droplet can be identified by such methods as the presence of dyes of multiple colors or the increase in the intensity of a single dye or a combination of both. Any method that allows the identification of multiple variants in a single droplet enables the determination of a samples haplotype.

In accordance with some implementations of the invention, a method is provided for analyzing a target nucleic acid that includes compartmentalizing a first fluid into portions, each portion containing a single target nucleic acid; amplifying the target in the portions; excluding portions containing amplicon from the target and amplicon from a variant of the target; and analyzing target amplicons.

In other aspects, the invention generally provides methods for detecting a recurrence of a cancer in a patient. Those methods may involve forming sample droplets containing a single target nucleic acid derived from a patient sample, flowing the sample droplets through a channel, amplifying the target in the droplets, detecting amplified target in the droplets, excluding droplets including a heterogeneous population of amplicons, and analyzing non-excluded droplets to determine the presence of mutant alleles indicative of recurrence. In certain embodiments, the analyzing step includes capturing amplicon obtained from the droplets using labeled capture probes. The sample may be a human tissue or body fluid. Exemplary body fluids are pus, sputum, semen, urine, blood, saliva, stool, and cerebrospinal fluid. In other aspects of the invention generally provide a method for forensic identification of low levels of target nucleic acid in an environment having multiple other sources of nucleic acid. Such methods may also be practiced using fluids compartmentalized in containers other than or in addition to droplets.

Brief Description of the Drawings

Figure 1 depicts a droplet formation device.

Figure 2 depicts a portion of the droplet formation device of Figure 1.

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Figure 3 depicts an exemplary microfluidic system for droplet generation and readout. Figure 3a depicts the droplet generation chip; Figure 3b depicts the droplet spacing for readout; and Figure 3c depicts a cartoon of droplet readout by fluorescence.

Figure 4 depicts the serial dilution of template DNA quantified by dPCR. Figure 4a shows droplet fluorescence during readout for the most concentrated sample. Each discrete burst of fluorescence corresponded to an individual droplet. Two different groups of droplets were evident: PCR(+) droplets peaking at ~0.8 V and PCR(-) droplets at ~0.1 V; Figure 4b shows a histogram of the peak fluorescence intensities of droplets from the complete data trace in (a). PCR(+) and PCR(-) droplets appeared as two very distinct populations centered at 0.78 and 0.10 V, respectively; Figure 4c shows the serial dilution of template DNA. Open circles: measured occupancies; solid line: the best fit to Eqn 2 ($A = 0.15, f = 4.8, R^2 - 0.9999$).

Figure 5A is a schematic representation of a droplet having 5 sets of primers for PCR amplification of a template sequence and 5 probes, each labeled with a fluorescent dye, that binds specifically to the amplified sequences; Figure 5B is a time trace of fluorescence intensity detected from droplets after PCR amplification; Figure 5C is a scatter plot showing clusters representing droplets that contain specific amplified sequences (TERT, RNaseP, E1a, SMN1 and SMN2).

Figure 6A is a schematic representation of a droplet having 5 sets of primers for PCR amplification of a template sequence and 5 probes, each labeled with a fluorescent dye, that binds specifically to the amplified sequences; Figure 6B is a scatter plot showing clusters representing droplets that contain specific amplified sequences (TERT, 815A, RNaseP, E1a, and 815G); Figure 6C is a table showing the copy number of specific sequences shown in Figure 6B.

Figure 7 is a schematic depicting one-color detection of a genetic sequence with a microfluidic device.

Figure 8 is a schematic depicting two-color detection of two genetic sequences with a microfluidic device.

Figure 9 is a schematic depicting two-color detection of three genetic sequences with a microfluidic device.

Figure 10 shows two dot plots depicting clusters of genetic sequences detected through fluorescence intensity. Left panel is a dot plot showing four clusters. Block for SMN1 sequence

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was present. Top left: microdroplets containing the reference sequence (SMARCC1); bottom left: microdroplets not containing any sequence; bottom middle: microdroplets containing sequence for SMN1; and bottom right: microdroplets containing sequence for SMN2. Right panel is a dot plot showing four clusters. No block for SMN1 sequence was present. Top left: microdroplets containing the reference sequence (SMARCC1); bottom left: microdroplets not containing any sequence; bottom middle: microdroplets containing sequence for SMN1; and bottom right: microdroplets containing sequence for SMN2. The shift of the bottom middle cluster in right panel as compared to left panel confirms that fluorescence intensity provides a very sensitive measurement for the presence of a sequence.

Figure 11 depicts histograms of a duplex gene copy number assay using only one type of fluorophore by digital PCR; Figure 11a depicts a histogram of droplet peak fluorescence intensities; Figure 11b shows a comparison of gene copy numbers measured by monochromatic dPCR.

Figure 12 is a schematic for tuning the intensity of a detectable label to a particular target with a microfluidic device.

Figure 13 is a line graph depicting the linear dependence of droplet fluorescence intensity on probe concentration (Line, best linear fit ($y = - 0.092x + 0.082$, $R^2 = 0.995$).

Figure 14 depicts a 5-plex dPCR assay for spinal muscular atrophy with only two fluorophores. Figure 14a is a 2D histogram of droplet fluorescence intensities, shown as a heat map, for the 5-plex assay against the synthetic model chromosome for validation. The six well resolved droplet populations corresponded to the five individual assays plus the empty droplets; Figure 14b shows the results of the SMA pilot study.

Figure 15 depicts a 9-plex dPCR assay for spinal muscular atrophy with only two fluorophores, showing the process of optimizing droplet intensities. Figures 15a and 15 b show 2-D histograms of droplet fluorescence intensity, shown as heat maps with hotter colors representing higher droplet counts, for the 9-plex assay against the synthetic model chromosome ((a) = Before optimization; (b) = after optimization).

Figure 16 depicts an optical schematic for combining optical labels with multiplexing.

Figure 17 depicts a dPCR assay combining multiplexing with optical labels using co-flow microfluidics. The contributions from all droplets are shown, that is, from three different triplex

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assays. (Both panels) 2-D histograms shown as heat maps with hotter colors representing higher droplet counts. (Left panel) histogram of optical labels, i.e. fluorescence intensities of droplets measured at wavelengths for the two fluorophores comprising the optical labels. (Right panel) assay histogram, i.e. fluorescence intensities of droplets measured at wavelengths suitable for FAM detection (x-axis), and VIC detection (y-axis). Both histograms were compensated for spectral overlap by standard techniques.

Figure 18 shows single assay selections using optical labels. Selections were taken from all of the droplets from Figure 17. Each of the three different selections in panels A–C were for optical labels encoding the same assay (TERT, SMN1, and SMN2). Histograms are as described in Figure 17. (Left histograms, optical labels) Superimposed lines demark the bounding box for selecting a single optical label. (Right histograms, assay) Only droplets containing the selected optical label are displayed.

Figure 19 shows single assay selections using optical labels. Selections were taken from all of the droplets from Figure17. Each of the three different selections in panels A–C was for optical labels encoding the same assay (TERT, c.5C from SMN1, and BCKDHA). Histograms are as described in Figure 17. (Left histograms, optical labels) Superimposed lines demark the bounding box for selecting a single optical label. (Right histograms, assay) Only droplets containing the selected optical label are displayed.

Figure 20 shows single assay selections using optical labels. Selections were taken from all of the droplets from Figure 17. Each of the three different selections in panels A–C was for optical labels encoding the same assay (TERT, c.88G from SMN1, and RNaseP). Histograms are as described in Figure 17. (Left histograms, optical labels) Superimposed lines demark the bounding box for selecting a single optical label. (Right histograms, assay) Only droplets containing the selected optical label are displayed.

Figure 21 depicts a dPCR assay combining multiplexing with optical labels using droplet merging.

Figure 22 is a schematic showing haplotype detection in droplets.

Detailed Description

The invention provides materials and methods for analysis of biomolecules. In one aspect, the invention provides for digital analysis in droplets, such as microfluidic droplets. The

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invention allows digital PCR to be conducted and provides for significantly reduced or eliminated errors.

Ideally, the sensitivity of digital PCR is limited only by the number of independent amplifications that can be analyzed, which has motivated the development of several ultra-high throughput miniaturized methods allowing millions of single molecule PCR reactions to be performed in parallel (discussed in detail elsewhere). In a preferred embodiment of the invention, digital PCR is performed in aqueous droplets separated by oil using a microfluidics system. In another preferred embodiment, the oil is a fluorinated oil such as the Fluorinert oils (3M). In a still more preferred embodiment the fluorinated oil contains a surfactant, such as PFPE-PEG-PFPE triblock copolymer, to stabilize the droplets against coalescence during the amplification step or at any point where they contact each other. Microfluidic approaches allow the rapid generation of large numbers (e.g. 10^6 or greater) of very uniformly sized droplets that function as picoliter volume reaction vessels (see reviews of droplet-based microfluidics). But as will be described, the invention is not limited to dPCR performed in water-in-oil emulsions, but rather is general to all methods of reaction compartmentalization for dPCR. In the description that follows, the invention is described in terms of the use of droplets for compartmentalization, but it is understood that this choice of description is not limiting for the invention, and that all of the methods of the invention are compatible with all other methods of reaction compartmentalization for dPCR.

Methods of the invention involve novel strategies for performing multiple different amplification reactions on the same sample simultaneously to quantify the abundance of multiple different DNA targets, commonly known to those familiar with the art as "multiplexing". Methods of the invention for multiplexing dPCR assays promise greater plexity—the number of simultaneous reactions—than possible with existing qPCR or dPCR techniques. It is based on the singular nature of amplifications at terminal or limiting dilution that arises because most often only a single target allele is ever present in any one droplet even when multiple primers/probes targeting different alleles are present. This alleviates the complications that otherwise plague simultaneous competing reactions, such as varying arrival time into the exponential stage and unintended interactions between primers.

In one aspect, the invention provides materials and methods for improving amplicon yield while maintaining the sensitivity and specificity in droplet based digital PCR. More

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specifically, the invention provides droplets containing a single nucleic acid template and multiplexed PCR primers and methods for detecting a plurality of targets in a biological sample by forming such droplets and amplifying the nucleic acid templates using droplet based digital PCR.

Reactions within microfluidic droplets yield very uniform fluorescence intensity at the end point, and ultimately the intensity depends on the efficiency of probe hydrolysis. Thus, in another aspect of the methods of the invention, different reactions with different efficiencies can be discriminated on the basis of end point fluorescence intensity alone even if they have the same color. Furthermore, in another method of the invention, the efficiencies can be tuned simply by adjusting the probe concentration, resulting in an easy-to-use and general purpose method for multiplexing. In one demonstration of the invention, a 5-plex TaqMan® dPCR assay worked "right out of the box", in contrast to lengthy optimizations that typify qPCR multiplexing to this degree. In another aspect of the invention, adding multiple colors increases the number of possible reactions geometrically, rather than linearly as with qPCR, because individual reactions can be labeled with multiple fluorophores. As an example, two fluorophores (VIC and FAM) were used to distinguish five different reactions in one implementation of the invention.

Methods of the invention are able to detect polymerase errors that occur during an amplification reaction and are able to exclude from analysis those products that are a result of polymerase errors. In essence, methods of the invention increase the sensitivity of digital PCR by identifying amplification products that are false positives, and excluding those products from analysis.

Methods of the invention involve forming sample droplets containing a single target nucleic acid, amplifying the target in the droplets, excluding droplets containing amplicon from the target and amplicon from a variant of the target, and analyzing target amplicons.

Nucleic Acid Target Molecules

Nucleic acid molecules include deoxyribonucleic acid (DNA) and/or ribonucleic acid (RNA). Nucleic acid molecules can be synthetic or derived from naturally occurring sources. In one embodiment, nucleic acid molecules are isolated from a biological sample containing a variety of other components, such as proteins, lipids and non-template nucleic acids. Nucleic acid template molecules can be obtained from any cellular material, obtained from an animal,

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plant, bacterium, fungus, or any other cellular organism. In certain embodiments, the nucleic acid molecules are obtained from a single cell. Biological samples for use in the present invention include viral particles or preparations. Nucleic acid molecules can be obtained directly from an organism or from a biological sample obtained from an organism, e.g., from blood, urine, cerebrospinal fluid, seminal fluid, saliva, sputum, stool and tissue. Any tissue or body fluid specimen may be used as a source for nucleic acid for use in the invention. Nucleic acid molecules can also be isolated from cultured cells, such as a primary cell culture or a cell line. The cells or tissues from which template nucleic acids are obtained can be infected with a virus or other intracellular pathogen. A sample can also be total RNA extracted from a biological specimen, a cDNA library, viral, or genomic DNA. In certain embodiments, the nucleic acid molecules are bound as to other target molecules such as proteins, enzymes, substrates, antibodies, binding agents, beads, small molecules, peptides, or any other molecule and serve as a surrogate for quantifying and / or detecting the target molecule.

Generally, nucleic acid can be extracted from a biological sample by a variety of techniques such as those described by Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y., pp. 280-281 (1982). Nucleic acid molecules may be single-stranded, double-stranded, or double-stranded with single-stranded regions (for example, stem- and loop-structures).

Droplet Formation

Methods of the invention involve forming sample droplets where some droplets contain zero target nucleic acid molecules, some droplets contain one target nucleic acid molecule, and some droplets may or may not contain multiple nucleic acid molecules (corresponding to limiting or terminal dilution, respectively, as defined above). In the preferred embodiment, the distribution of molecules within droplets obeys the Poisson distribution. However, methods for non-Poisson loading of droplets are known to those familiar with the art, and include but are not limited to active sorting of droplets, such as by laser-induced fluorescence, or by passive one-to-one loading. The description that follows assumes Poisson loading of droplets, but such description is not intended to exclude non-Poisson loading, as the invention is compatible with all distributions of DNA loading that conform to limiting or terminal dilution.

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The droplets are aqueous droplets that are surrounded by an immiscible carrier fluid. Methods of forming such droplets are shown for example in Link et al. (U.S. patent application numbers 2008/0014589, 2008/0003142, and 2010/0137163), Stone et al. (U.S. patent number 7,708,949 and U.S. patent application number 2010/0172803), Anderson et al. (U.S. patent number 7,041,481 and which reissued as RE41,780) and European publication number EP2047910 to Raindance Technologies Inc. The content of each of which is incorporated by reference herein in its entirety.

Figure 1 shows an exemplary embodiment of a device **100** for droplet formation. Device **100** includes an inlet channel **101**, and outlet channel **102**, and two carrier fluid channels **103** and **104**. Channels **101**, **102**, **103**, and **104** meet at a junction **105**. Inlet channel **101** flows sample fluid to the junction **105**. Carrier fluid channels **103** and **104** flow a carrier fluid that is immiscible with the sample fluid to the junction **105**. Inlet channel **101** narrows at its distal portion wherein it connects to junction **105** (See Figure 2). Inlet channel **101** is oriented to be perpendicular to carrier fluid channels **103** and **104**. Droplets are formed as sample fluid flows from inlet channel **101** to junction **105**, where the sample fluid interacts with flowing carrier fluid provided to the junction **105** by carrier fluid channels **103** and **104**. Outlet channel **102** receives the droplets of sample fluid surrounded by carrier fluid.

The sample fluid is typically an aqueous buffer solution, such as ultrapure water (e.g., 18 mega-ohm resistivity, obtained, for example by column chromatography), 10 mM Tris HCl and 1 mM EDTA (TE) buffer, phosphate buffer saline (PBS) or acetate buffer. Any liquid or buffer that is physiologically compatible with nucleic acid molecules can be used. The carrier fluid is one that is immiscible with the sample fluid. The carrier fluid can be a non-polar solvent, decane (e g., tetradecane or hexadecane), fluorocarbon oil, silicone oil or another oil (for example, mineral oil).

In certain embodiments, the carrier fluid contains one or more additives, such as agents which increase, reduce, or otherwise create non-Newtonian surface tensions (surfactants) and/or stabilize droplets against spontaneous coalescence on contact. Surfactants can include Tween, Span, fluorosurfactants, and other agents that are soluble in oil relative to water. In some applications, performance is improved by adding a second surfactant, or other agent, such as a polymer or other additive, to the sample fluid. Surfactants can aid in controlling or optimizing droplet size, flow and uniformity, for example by reducing the shear force needed to extrude or

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inject droplets into an intersecting channel. This can affect droplet volume and periodicity, or the rate or frequency at which droplets break off into an intersecting channel. Furthermore, the surfactant can serve to stabilize aqueous emulsions in fluorinated oils from coalescing.

In certain embodiments, the droplets may be coated with a surfactant or a mixture of surfactants. Preferred surfactants that may be added to the carrier fluid include, but are not limited to, surfactants such as sorbitan-based carboxylic acid esters (e.g., the "Span" surfactants, Fluka Chemika), including sorbitan monolaurate (Span 20), sorbitan monopalmitate (Span 40), sorbitan monostearate (Span 60) and sorbitan monooleate (Span 80), and perfluorinated polyethers (e.g., DuPont Krytox 157 FSL, FSM, and/or FSH). Other non-limiting examples of non-ionic surfactants which may be used include polyoxyethylenated alkylphenols (for example, nonyl-, p-dodecyl-, and dinonylphenols), polyoxyethylenated straight chain alcohols, polyoxyethylenated polyoxypropylene glycols, polyoxyethylenated mercaptans, long chain carboxylic acid esters (for example, glyceryl and polyglycerl esters of natural fatty acids, propylene glycol, sorbitol, polyoxyethylenated sorbitol esters, polyoxyethylene glycol esters, etc.) and alkanolamines (e.g., diethanolamine-fatty acid condensates and isopropanolamine-fatty acid condensates).

In certain embodiments, the carrier fluid may be caused to flow through the outlet channel so that the surfactant in the carrier fluid coats the channel walls. In one embodiment, the fluorosurfactant can be prepared by reacting the perflourinated polyether DuPont Krytox 157 FSL, FSM, or FSH with aqueous ammonium hydroxide in a volatile fluorinated solvent. The solvent and residual water and ammonia can be removed with a rotary evaporator. The surfactant can then be dissolved (e.g., 2.5 wt %) in a fluorinated oil (e.g., Flourinert (3M)), which then serves as the carrier fluid.

One approach to merging sample fluids, using a device called a lambda injector, involves forming a droplet, and contacting the droplet with a fluid stream, in which a portion of the fluid stream integrates with the droplet to form a mixed droplet. In this approach, only one phase needs to reach a merge area in a form of a droplet. Further description of such method is shown in the co-owned and co-pending U.S. patent application to Yurkovetsky, et al. (U.S. patent application serial number 61/441,985), the content of which is incorporated y reference herein in its entirety.

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According to a method for operating the lambda injector, a droplet is formed as described above. After formation of the sample droplet from the first sample fluid, the droplet is contacted with a flow of a second sample fluid stream. Contact between the droplet and the fluid stream results in a portion of the fluid stream integrating with the droplet to form a mixed droplet.

The droplets of the first sample fluid flow through a first channel separated from each other by immiscible carrier fluid and suspended in the immiscible carrier fluid. The droplets are delivered to the merge area, i.e., junction of the first channel with the second channel, by a pressure-driven flow generated by a positive displacement pump. While droplet arrives at the merge area, a bolus of a second sample fluid is protruding from an opening of the second channel into the first channel. Preferably, the channels are oriented perpendicular to each other. However, any angle that results in an intersection of the channels may be used.

The bolus of the second sample fluid stream continues to increase in size due to pumping action of a positive displacement pump connected to channel, which outputs a steady stream of the second sample fluid into the merge area. The flowing droplet containing the first sample fluid eventually contacts the bolus of the second sample fluid that is protruding into the first channel. Contact between the two sample fluids results in a portion of the second sample fluid being segmented from the second sample fluid stream and joining with the first sample fluid droplet to form a mixed droplet. In certain embodiments, each incoming droplet of first sample fluid is merged with the same amount of second sample fluid.

In certain embodiments, an electric charge is applied to the first and second sample fluids. Description of applying electric charge to sample fluids is provided in Link et al. (U.S. patent application number 2007/0003442) and European Patent Number EP2004316 to Raindance Technologies Inc, the content of each of which is incorporated by reference herein in its entirety. Electric charge may be created in the first and second sample fluids within the carrier fluid using any suitable technique, for example, by placing the first and second sample fluids within an electric field (which may be AC, DC, etc.), and/or causing a reaction to occur that causes the first and second sample fluids to have an electric charge, for example, a chemical reaction, an ionic reaction, a photocatalyzed reaction, etc.

The electric field, in some embodiments, is generated from an electric field generator, i.e., a device or system able to create an electric field that can be applied to the fluid. The electric field generator may produce an AC field (i.e., one that varies periodically with respect to time,

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for example, sinusoidally, sawtooth, square, etc.), a DC field (i.e., one that is constant with respect to time), a pulsed field, etc. The electric field generator may be constructed and arranged to create an electric field within a fluid contained within a channel or a microfluidic channel. The electric field generator may be integral to or separate from the fluidic system containing the channel or microfluidic channel, according to some embodiments.

Techniques for producing a suitable electric field (which may be AC, DC, etc.) are known to those of ordinary skill in the art. For example, in one embodiment, an electric field is produced by applying voltage across a pair of electrodes, which may be positioned on or embedded within the fluidic system (for example, within a substrate defining the channel or microfluidic channel), and/or positioned proximate the fluid such that at least a portion of the electric field interacts with the fluid. The electrodes can be fashioned from any suitable electrode material or materials known to those of ordinary skill in the art, including, but not limited to, silver, gold, copper, carbon, platinum, copper, tungsten, tin, cadmium, nickel, indium tin oxide ("ITO"), etc., as well as combinations thereof. In some cases, transparent or substantially transparent electrodes can be used.

The electric field facilitates rupture of the interface separating the second sample fluid and the droplet. Rupturing the interface facilitates merging of bolus of the second sample fluid and the first sample fluid droplet. The forming mixed droplet continues to increase in size until it a portion of the second sample fluid breaks free or segments from the second sample fluid stream prior to arrival and merging of the next droplet containing the first sample fluid. The segmenting of the portion of the second sample fluid from the second sample fluid stream occurs as soon as the shear force exerted on the forming mixed droplet by the immiscible carrier fluid overcomes the surface tension whose action is to keep the segmenting portion of the second sample fluid connected with the second sample fluid stream. The now fully formed mixed droplet continues to flow through the first channel.

In other embodiments, the rupture of the interface can be spontaneous, or the rupture can be facilitated by surface chemistry. The invention is not limited in regard to the method of rupture at the interface, as rupture can be brought about by any means.

In the context of PCR, in a preferred embodiment, the first sample fluid contains nucleic acid templates. Droplets of the first sample fluid are formed as described above. Those droplets will include the nucleic acid templates. In certain embodiments, the droplets will include only a

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single nucleic acid template, and thus digital PCR can be conducted. The second sample fluid contains reagents for the PCR reaction. Such reagents generally include Taq polymerase, deoxynucleotides of type A, C, G and T, magnesium chloride, and forward and reverse primers, all suspended within an aqueous buffer. The second fluid also includes detectably labeled probes for detection of the amplified target nucleic acid, the details of which are discussed below. A droplet containing the nucleic acid is then caused to merge with the PCR reagents in the second fluid as described above, producing a droplet that includes Taq polymerase, deoxynucleotides of type A, C, G and T, magnesium chloride, forward and reverse primers, detectably labeled probes, and the target nucleic acid. In another embodiment, the first fluid can contain the template DNA and PCR master mix (defined below), and the second fluid can contain the forward and reverse primers and the probe. The invention is not restricted in any way regarding the constituency of the first and second fluidics for PCR or digital PCR. For example, in some embodiments, the template DNA is contained in the second fluid inside droplets.

Target Amplification

Methods of the invention further involve amplifying the target nucleic acid in each droplet. Amplification refers to production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction or other technologies well known in the art (e.g., Dieffenbach and Dveksler, PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y. [1995]). The amplification reaction may be any amplification reaction known in the art that amplifies nucleic acid molecules, such as polymerase chain reaction, nested polymerase chain reaction, ligase chain reaction (Barany F. (1991) PNAS 88:189-193; Barany F. (1991) PCR Methods and Applications 1:5-16), ligase detection reaction (Barany F. (1991) PNAS 88:189-193), strand displacement amplification, transcription based amplification system, nucleic acid sequence-based amplification, rolling circle amplification, and hyper-branched rolling circle amplification.

In certain embodiments, the amplification reaction is the polymerase chain reaction. Polymerase chain reaction (PCR) refers to methods by K. B. Mullis (U.S. patent numbers 4,683,195 and 4,683,202, hereby incorporated by reference) for increasing concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. The process for amplifying the target sequence includes introducing an excess of oligonucleotide

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primers to a DNA mixture containing a desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The primers are complementary to their respective strands of the double stranded target sequence.

To effect amplification, primers are annealed to their complementary sequence within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension can be repeated many times (i.e., denaturation, annealing and extension constitute one cycle; there can be numerous cycles) to obtain a high concentration of an amplified segment of a desired target sequence. The length of the amplified segment of the desired target sequence is determined by relative positions of the primers with respect to each other and by cycling parameters, and therefore, this length is a controllable parameter.

Methods for performing PCR in droplets are shown for example in Link et al. (U.S. patent application numbers 2008/0014589, 2008/0003142, and 2010/0137163), Anderson et al. (U.S. patent number 7,041,481 and which reissued as RE41,780) and European publication number EP2047910 to Raindance Technologies Inc. The content of each of which is incorporated by reference herein in its entirety.

The sample droplet may be pre-mixed with a primer or primers, or the primer or primers may be added to the droplet. In some embodiments, droplets created by segmenting the starting sample are merged with a second set of droplets including one or more primers for the target nucleic acid in order to produce final droplets. The merging of droplets can be accomplished using, for example, one or more droplet merging techniques described for example in Link et al. (U.S. patent application numbers 2008/0014589, 2008/0003142, and 2010/0137163) and European publication number EP2047910 to Raindance Technologies Inc.

In embodiments involving merging of droplets, two droplet formation modules are used. In one embodiment, a first droplet formation module produces the sample droplets consistent with limiting or terminal dilution of target nucleic acid. A second droplet formation or reinjection module inserts droplets that contain reagents for a PCR reaction. Such droplets generally include the "PCR master mix" (known to those in the art as a mixture containing at least Taq polymerase, deoxynucleotides of type A, C, G and T, and magnesium chloride) and forward and reverse primers (known to those in the art collectively as "primers"), all suspended within an aqueous buffer. The second droplet also includes detectably labeled probes for

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detection of the amplified target nucleic acid, the details of which are discussed below. Different arrangements of reagents between the two droplet types is envisioned. For example, in another embodiment, the template droplets also contain the PCR master mix, but the primers and probes remain in the second droplets. Any arrangement of reagents and template DNA can be used according to the invention.

Primers can be prepared by a variety of methods including but not limited to cloning of appropriate sequences and direct chemical synthesis using methods well known in the art (Narang et al., Methods Enzymol., 68:90 (1979); Brown et al., Methods Enzymol., 68:109 (1979)). Primers can also be obtained from commercial sources such as Operon Technologies, Amersham Pharmacia Biotech, Sigma, and Life Technologies. The primers can have an identical melting temperature. The lengths of the primers can be extended or shortened at the 5' end or the 3' end to produce primers with desired melting temperatures. Also, the annealing position of each primer pair can be designed such that the sequence and, length of the primer pairs yield the desired melting temperature. The simplest equation for determining the melting temperature of primers smaller than 25 base pairs is the Wallace Rule (Td=2(A+T)+4(G+C)). Another method for determining the melting temperature of primers is the nearest neighbor method Computer programs can also be used to design primers, including but not limited to Array Designer Software (Arrayit Inc.), Oligonucleotide Probe Sequence Design Software for Genetic Analysis (Olympus Optical Co.), NetPrimer, and DNAsis from Hitachi Software Engineering. The TM (melting or annealing temperature) of each primer is calculated using software programs such as Oligo Design, available from Invitrogen Corp.

In one embodiment, the droplet formation modules are arranged and controlled to produce an interdigitation of sample droplets and PCR reagent droplets flowing through a channel. Such an arrangement is described for example in Link et al. (U.S. patent application numbers 2008/0014589, 2008/0003142, and 2010/0137163) and European publication number EP2047910 to Raindance Technologies Inc.

A sample droplet is then caused to merge with a PCR reagent droplet, producing a droplet that includes the PCR master mix, primers, detectably labeled probes, and the target nucleic acid. Droplets may be merged for example by: producing dielectrophoretic forces on the droplets using electric field gradients and then controlling the forces to cause the droplets to merge; producing droplets of different sizes that thus travel at different velocities, which causes the

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droplets to merge; and producing droplets having different viscosities that thus travel at different velocities, which causes the droplets to merge with each other. Each of those techniques is further described in Link et al. (U.S. patent application numbers 2008/0014589, 2008/0003142, and 2010/0137163) and European publication number EP2047910 to Raindance Technologies Inc. Further description of producing and controlling dielectrophoretic forces on droplets to cause the droplets to merge is described in Link et al. (U.S. patent application number 2007/0003442) and European Patent Number EP2004316 to Raindance Technologies Inc.

In another embodiment, called simple droplet generation, a single droplet formation module, or a plurality of droplet formation modules are arranged to produce droplets from a mixture already containing the template DNA, the PCR master mix, primers, and detectably labeled probes. In yet another embodiment, called co-flow, upstream from a single droplet formation module two channels intersect allowing two flow streams to converge. One flow stream contains one set of reagents and the template DNA, and the other contains the remaining reagents. In the preferred embodiment for co-flow, the template DNA and the PCR master mix are in one flow stream, and the primers and probes are in the other. However, the invention is not limited in regard to the constituency of either flow stream. For example, in another embodiment, one flow stream contains just the template DNA, and the other contains the PCR master mix, the primers, and the probes. On convergence of the flow streams in a fluidic intersection, the flow streams may or may not mix before the droplet generation nozzle. In either embodiment, some amount of fluid from the first stream, and some amount of fluid from the second stream are encapsulated within a single droplet. Following encapsulation, complete mixing occurs.

Once final droplets have been produced by any of the droplet forming embodiments above, or by any other embodiments, the droplets are thermal cycled, resulting in amplification of the target nucleic acid in each droplet. In certain embodiments, the droplets are collected off-chip as an emulsion in a PCR thermal cycling tube and then thermally cycled in a conventional thermal cycler. Temperature profiles for thermal cycling can be adjusted and optimized as with any conventional DNA amplification by PCR.

In certain embodiments, the droplets are flowed through a channel in a serpentine path between heating and cooling lines to amplify the nucleic acid in the droplet. The width and

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depth of the channel may be adjusted to set the residence time at each temperature, which can be controlled to anywhere between less than a second and minutes.

In certain embodiments, the three temperature zones are used for the amplification reaction. The three temperature zones are controlled to result in denaturation of double stranded nucleic acid (high temperature zone), annealing of primers (low temperature zones), and amplification of single stranded nucleic acid to produce double stranded nucleic acids (intermediate temperature zones). The temperatures within these zones fall within ranges well known in the art for conducting PCR reactions. See for example, Sambrook et al. (Molecular Cloning, A Laboratory Manual, 3rd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001).

In certain embodiments, the three temperature zones are controlled to have temperatures as follows: 95^0C (T_H), 55^0C (T_L), 72^0C (T_M). The prepared sample droplets flow through the channel at a controlled rate. The sample droplets first pass the initial denaturation zone (T_H) before thermal cycling. The initial preheat is an extended zone to ensure that nucleic acids within the sample droplet have denatured successfully before thermal cycling. The requirement for a preheat zone and the length of denaturation time required is dependent on the chemistry being used in the reaction. The samples pass into the high temperature zone, of approximately 95^0C, where the sample is first separated into single stranded DNA in a process called denaturation. The sample then flows to the low temperature, of approximately 55^0C, where the hybridization process takes place, during which the primers anneal to the complementary sequences of the sample. Finally, as the sample flows through the third medium temperature, of approximately 72^0C, the polymerase process occurs when the primers are extended along the single strand of DNA with a thermostable enzyme. Methods for controlling the temperature in each zone may include but are not limited to electrical resistance, peltier junction, microwave radiation, and illumination with infrared radiation.

The nucleic acids undergo the same thermal cycling and chemical reaction as the droplets passes through each thermal cycle as they flow through the channel. The total number of cycles in the device is easily altered by an extension of thermal zones or by the creation of a continuous loop structure. The sample undergoes the same thermal cycling and chemical reaction as it passes through N amplification cycles of the complete thermal device.

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In other embodiments, the temperature zones are controlled to achieve two individual temperature zones for a PCR reaction. In certain embodiments, the two temperature zones are controlled to have temperatures as follows: 95^0C (T_H) and 60^0C (T_L). The sample droplet optionally flows through an initial preheat zone before entering thermal cycling. The preheat zone may be important for some chemistry for activation and also to ensure that double stranded nucleic acid in the droplets are fully denatured before the thermal cycling reaction begins. In an exemplary embodiment, the preheat dwell length results in approximately 10 minutes preheat of the droplets at the higher temperature.

The sample droplet continues into the high temperature zone, of approximately 95^0C, where the sample is first separated into single stranded DNA in a process called denaturation. The sample then flows through the device to the low temperature zone, of approximately 60^0C, where the hybridization process takes place, during which the primers anneal to the complementary sequences of the sample. Finally the polymerase process occurs when the primers are extended along the single strand of DNA with a thermostable enzyme. The sample undergoes the same thermal cycling and chemical reaction as it passes through each thermal cycle of the complete device. The total number of cycles in the device is easily altered by an extension of block length and tubing.

In another embodiment the droplets are created and/or merged on chip followed by their storage either on the same chip or another chip or off chip in some type of storage vessel such as a PCR tube. The chip or storage vessel containing the droplets is then cycled in its entirety to achieve the desired PCR heating and cooling cycles.

In another embodiment the droplets are collected in a chamber where the density difference between the droplets and the surrounding oil allows for the oil to be rapidly exchanged without removing the droplets. The temperature of the droplets can then be rapidly changed by exchange of the oil in the vessel for oil of a different temperature. This technique is broadly useful with two and three step temperature cycling or any other sequence of temperatures.

The invention is not limited by the method used to thermocycle the droplets. Any method of thermocycling the droplets may be used.

Target Detection

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After amplification, droplets are flowed to a detection module for detection of amplification products. For embodiments in which the droplets are thermally cycled off-chip, the droplets require re-injection into either a second fluidic circuit for read-out—that may or may not reside on the same chip as the fluidic circuit or circuits for droplet generation—or in certain embodiments the droplets may be reinjected for read-out back into the original fluidic circuit used for droplet generation. The droplets may be individually analyzed and detected using any methods known in the art, such as detecting the presence or amount of a reporter. Generally, the detection module is in communication with one or more detection apparatuses. The detection apparatuses can be optical or electrical detectors or combinations thereof. Examples of suitable detection apparatuses include optical waveguides, microscopes, diodes, light stimulating devices, (e.g., lasers), photo multiplier tubes, and processors (e.g., computers and software), and combinations thereof, which cooperate to detect a signal representative of a characteristic, marker, or reporter, and to determine and direct the measurement or the sorting action at a sorting module. Further description of detection modules and methods of detecting amplification products in droplets are shown in Link et al. (U.S. patent application numbers 2008/0014589, 2008/0003142, and 2010/0137163) and European publication number EP2047910 to Raindance Technologies Inc.

In certain embodiments, amplified target are detected using detectably labeled probes. In particular embodiments, the detectably labeled probes are optically labeled probes, such as fluorescently labeled probes. Examples of fluorescent labels include, but are not limited to, Atto dyes, 4-acetamido-4'-isothiocyanatostilbene-2,2'disulfonic acid; acridine and derivatives: acridine, acridine isothiocyanate; 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS); 4-amino-N-[3-vinylsulfonyl)phenyl]naphthalimide-3,5 disulfonate; N-(4-anilino-1-naphthyl)maleimide; anthranilamide; BODIPY; Brilliant Yellow; coumarin and derivatives; coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifluoromethylcouluarin (Coumaran 151); cyanine dyes; cyanosine; 4',6-diaminidino-2-phenylindole (DAPI); 5'5"-dibromopyrogallol-sulfonaphthalein (Bromopyrogallol Red); 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; 5-[dimethylamino]naphthalene-1-sulfonyl chloride (DNS, dansylchloride); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives; eosin, eosin

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isothiocyanate, erythrosin and derivatives; erythrosin B, erythrosin, isothiocyanate; ethidium; fluorescein and derivatives; 5-carboxyfluorescein (FAM), 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), 2',7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein, fluorescein, fluorescein isothiocyanate, QFITC, (XRITC); fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferoneortho cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthaldialdehyde; pyrene and derivatives: pyrene, pyrene butyrate, succinimidyl 1-pyrene; butyrate quantum dots; Reactive Red 4 (Cibacron.TM. Brilliant Red 3B-A) rhodamine and derivatives: 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, sulforhodamine B, sulforhodamine 101, sulfonyl chloride derivative of sulforhodamine 101 (Texas Red); N,N,N',N'tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine isothiocyanate (TRITC); riboflavin; rosolic acid; terbium chelate derivatives; Cy3; Cy5; Cy5.5; Cy7; IRD 700; IRD 800; La Jolta Blue; phthalo cyanine; and naphthalo cyanine. Preferred fluorescent labels are FAM and VIC™ (from Applied Biosystems). Labels other than fluorescent labels are contemplated by the invention, including other optically-detectable labels.

In certain aspects, the droplets of the invention contain a plurality of detectable probes that hybridize to amplicons produced in the droplets. Members of the plurality of probes can each include the same detectable label, or a different detectable label. The plurality of probes can also include one or more groups of probes at varying concentration. The groups of probes at varying concentrations can include the same detectable label which vary in intensity, due to varying probe concentrations.

In a separate embodiment the detection can occur by the scanning of droplets confined to a monolayer in a storage device that is transparent to the wavelengths or method or detection. Droplets stored in this fashion can be scanned either by the movement of the storage device by the scanner or the movement of the scanner over the storage device.

The invention is not limited to the TaqMan assay, as described above, but rather the invention encompasses the use of all fluorogenic DNA hybridization probes, such as molecular beacons, Solaris probes, scorpion probes, and any other probes that function by sequence specific recognition of target DNA by hybridization and result in increased fluorescence on amplification of the target sequence.

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Digital PCR Performance in Droplets

Digital PCR performance in the emulsion format was validated by measuring a serial dilution of a reference gene, branched chain keto acid dehydrogenase E1 (BCKDHA). Mixtures of the PCR master mix, 1× primers and probe for BCKDHA, and varying concentrations of a mixture of human genomic DNA (1:1 NA14091 and NA13705) were compartmentalized into over one million 5.3 pL droplets in a water-in-fluorinated oil emulsion using the droplet generation microfluidic chip. The emulsion was thermally cycled off-chip and afterwards the fluorescence of each droplet was analyzed by fluorescence in the readout chip (see Fig. 3).

An exemplary microfluidic system for droplet generation and readout is depicted in Fig. 3. The microfluidic system for droplet generation and readout. As shown in Fig. 3a (droplet generation chip), a continuous aqueous phase containing the PCR master mix, primers, and probes, and template DNA flowed into the fluidic intersection from the left, and the carrier oil entered from the top and bottom. An emerging bolus of aqueous liquid was imaged inside the intersection just prior to snapping off into a discrete 4 pL droplet as the fluidic strain began to exceed the surface tension of the aqueous liquid. The steady train of droplets leaving the intersection toward the right was collected off chip as a stable emulsion for thermal cycling. Figure 3b depicts the droplet spacing for readout. Flows were arranged as in 3a, except instead of a continous phase, the emulsion from (a) was injected from the left into the intersection after thermal cycling. The oil drained from the emulsion during off-chip handling, hence the emulsion appeared tightly packed in the image before the intersection. The oil introduced in the intersection separated the droplets and the fluorescence of each droplet was measured at the location marked by the arrow. Figure 3c depicts a cartoon of droplet readout by fluorescence. The relatively infrequent PCR(+) droplets (light gray) flow along with the majority of PCR(-) droplets (dark gray) toward the detector. The droplets were interrogated sequentially by laser induced fluorescence while passing through the detection region.

In a serial dilution the average number of target DNA molecules per droplet—called the "occupancy" from this point forward—should decrease in direct proportion to the DNA concentration. The occupancy was calculated from Poisson statistics using the following equation well known to those experienced in the art:

$$\text{occupancy} = \ln\left(\frac{P+N}{N}\right), \quad (1)$$

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where P and N are the numbers of PCR(+) and PCR(–) droplets respectively.

Droplets were analyzed by fluorescence while flowing through the readout chip to count the numbers of PCR(+) and PCR(–) droplets (see Fig.3c). As each droplet passed the detection zone (marked with an arrow in Fig. 3b), a burst of fluorescence was observed. To account for small run-to-run differences in the fluorescence intensity that can occur due to different chip positioning, etc., each set of data was scaled such that the average fluorescence intensity of the empty droplets was 0.1 V. Fig. 4a shows a very short duration of a typical trace of fluorescence bursts from individual droplets for the sample with the highest DNA concentration in the series. PCR(+) and PCR(–) droplets were easily discriminated by fluorescence intensity. The two large bursts of fluorescence peaking at ~0.8 V arose from the PCR(+) droplets, whereas the smaller bursts due to incomplete fluorescence quenching in the PCR(–) droplets peaked at ~0.1 V. A histogram of peak intensities from the complete data set revealed two clear populations centered at 0.10 and 0.78 V (Fig. 4b), demonstrating that the trend evident in the short trace in Fig. 4a was stable over much longer periods of time. Integration over the two populations in Fig. 4b yielded a total of 197,507 PCR(+) and 1,240,126 PCR(–) droplets. Hence the occupancy was 0.15 for this sample by Eqn. 1, corresponding to the expected occupancy of 0.18 based on the measured DNA concentration of 110 ng/μL. The occupancy was measured for each sample in the serial dilution and fit to the dilution equation:

$$\text{occupancy}(n) = \frac{A}{f^n}, \tag{2}$$

where n is the number of dilutions, A is the occupancy at the starting concentration ($n = 0$), and f is the dilution factor. The linear fit was in excellent agreement with the data, with an R^2 value of 0.9999 and the fitted dilution factor of 4.8 in close agreement with the expected value of 5.0.

Multiplexing Primers in a Digital PCR Reaction

Droplet based digital PCR technology, as described in Link et al. (U.S. patent application numbers 2008/0014589, 2008/0003142, and 2010/0137163), Anderson et al. (U.S. patent number 7,041,481 and which reissued as RE41,780) and European publication number EP2047910 to Raindance Technologies Inc, (the contents of each of which are incorporated by reference herein in their entireties) utilizes a single primer pair per library droplet. This library droplet is merged with a template droplet which contains all the PCR reagents including genomic DNA except for the primers. After merging of the template and the primer library droplets the new droplet now

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contains all the reagents necessary to perform PCR. The droplet is then thermal cycled to produce amplicons. In one embodiment, the template DNA is diluted in the template mix such that on average there is less than one haploid genome per droplet.

Having only one haploid genome (i.e., one allele) per droplet gives droplet PCR advantages over standard singleplex or multiplex PCR in tubes or microwells. For example, in traditional PCR, both alleles are present in the reaction mix so if there is a difference in the PCR efficiency between alleles, the allele with the highest efficiency will be over represented. Additionally, there can be variances in the sequence to which the PCR primers hybridize, despite careful primer design. A variance in the primer hybridization sequence can cause that primer to have a lower efficiency for hybridization for the allele that has the variance compared to the allele that has the wild type sequence. This can also cause one allele to be amplified preferentially over the other allele if both alleles are present in the same reaction mix.

These issues are avoided in droplet based PCR because there is only one template molecule per droplet, and thus one allele per droplet. Thus, even if primer variance exists that reduces the PCR efficiency for one allele, there is no competition between alleles because the alleles are separated and thus uniformly amplified.

Optimization of traditional multiplexing of standard PCR primers in tubes or wells is known to be difficult. Multiple PCR amplicons being generated in the same reaction can lead to competition between amplicons that have differing efficiencies due to differences in sequence or length. This results in varying yields between competing amplicons which can result in non uniform amplicon yields. However, because droplet based digital PCR utilizes only one template molecule per droplet, even if there are multiple PCR primer pairs present in the droplet, only one primer pair will be active. Since only one amplicon is being generated per droplet, there is no competition between amplicons, resulting in a more uniform amplicon yield between different amplicons.

A certain amount of DNA is required to generate either a specific quantity of DNA and/or a specific number of PCR positive droplets to achieve sufficient sequencing coverage per base. Because only a percentage of the droplets are PCR positive, approximately 1 in 3 in the standard procedure, it takes more DNA to achieve the equivalent PCR yield per template DNA molecule. The number of PCR positive droplets and thus the amplicon yield can be increased by adding more genomic DNA. For instance, increasing the amount of genomic DNA twofold

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while maintaining the number of droplets constant will double the amplicon yield. However there is a limit to the amount of genomic DNA that can be added before there is a significant chance of having both alleles for a gene in the same droplet, thereby eliminating the advantage of droplet PCR for overcoming allele specific PCR and resulting in allelic dropout.

One way to allow the input of more genomic DNA is by generating more droplets to keep the haploid molecules per droplet ratio constant. For instance doubling the amount of DNA and doubling the amount of droplets increases the amplicon yield by 2x while maintaining the same haploid genome per droplet ratio. However, while doubling the number of droplets isn't problematic, increasing the amount of DNA can be challenging to users that have a limited amount of DNA.

The multiplexing of PCR primers in droplets enables the simultaneous increase in the number of PCR droplets while keeping the amount of input DNA the same or lower to generate an equal or greater amplicon yield. This results in an overall increase in the amount of PCR positive droplets and amplicon yield without the consumption of more DNA.

By way of example, if there is an average of 1 haploid genome per every 4 droplets or ¼ of the haploid genome per droplet and one PCR primer pair per droplet, the chances of the correct template being present for the PCR primer in the droplet is 1 out of 4. However, if there are 2 PCR primer pairs per droplet, then there is double the chance that there will be the correct template present in the droplet. This results in 1 out of 2 droplets being PCR positive which doubles the amplicon yield without doubling the input DNA. If the number of droplets containing the 2x multiplexed primers is doubled and the DNA kept constant, then the number of PCR positive droplets drops back to 1 in 4, but the total number of PCR droplets remains the same because the number of droplets have been doubled. If the multiplexing level in each droplet is increased to 4x and the input DNA is the same, the chance of the correct template molecule being present in each droplet doubles. This results in the number of PCR positive droplets being increased to 1 in 2 which doubles the amount of amplicon yield without increasing the amount of input DNA. Thus, by increasing the multiplexing of PCR primers in each droplet and by increasing the number of droplets overall, the amplicon yield can be increased by 4-fold without increasing the amount of input DNA.

Alternatively, if the amplicon yield is already sufficient, by increasing the multiplexing level for the PCR primers in each droplet, the amount of input genomic DNA can be dropped

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without sacrificing amplicon yield. For example if the multiplexing level of the PCR primers goes from 1x to 2x, the amount of input genomic DNA can be decreased by 2x while still maintaining the same overall amplicon yield.

Even though the number of PCR primer pairs per droplet is greater than one, there is still only one template molecule per droplet and thus there is only one primer pair per droplet that is being utilized at one time. This means that the advantages of droplet PCR for eliminating bias from either allele specific PCR or competition between different amplicons is maintained.

An example demonstration of droplet-based amplification and detection of multiple target sequences in a single droplet is shown here. Multiple copies of 5 sets of primers (primers for TERT, RNaseP, E1a, SMN1 and SMN2) were encapsulated in a single droplet at various concentrations along with the template DNA and the PCR master mix. Probes that specifically bind to TERT, RNaseP, E1a, SMN1 or SMN2 were also encapsulated in the droplets containing the primers. Probes for TERT, RNaseP and E1a were labeled with the VIC dye and probes for SMN1 and SMN2 were labeled with the FAM dye. The sequences for TERT RNaseP, E1a, SMN1 and SMN2 were amplified by PCR. The PCR was conducted with a standard thermal cycling setting. For example:

95 °C for 10 min
31 cycles
    92 °C for 15 s
    60 °C for 60 s

At the end of the PCR, the fluorescence emission from each droplet was determined and plotted on a scattered plot based on its wavelength and intensity. Six clusters, each representing droplets having the corresponding fluorescence wavelength and intensity were shown. The TERT, RNaseP and E1a clusters showed the fluorescence of the VIC dye at three distinct intensities and SMN1 and SMN1 clusters showed the fluorescence of the FAM dye at two distinct intensities (Figure 5). The number of droplets, each having one or more sequences selected from TERT, RNaseP, E1a, SMN1 and SMN2, can be determined from the scattered plot.

In an another demonstration of droplet-based amplification and detection of multiple target sequences in a single droplet, 5 sets of primers (primers for TERT, RNaseP, E1a, 815A and 815G) were encapsulated in a single droplet at various concentrations along with the

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template DNA, the PCR master mix, and the probes. The five different probes TERT, RNaseP, E1a, 815A and 815G were also encapsulated in the droplets containing the primers. Probes for TERT and 815A were labeled with the VIC dye and probes for 815G were labeled with the FAM dye. For each of RNaseP and E1a, two probes, one labeled with the VIC dye and the other labeled with the FAM dye, were encapsulated.

The droplets containing both the primers and probes were fused with droplets containing the template. PCR reactions were conducted with the fused droplets to amply the sequences for TERT, RNaseP, E1a, 815A and 815G. The PCR was conducted with a standard thermal cycling setting.

At the end of the PCR, the fluorescence emission from each fused droplet was determined and plotted on a scattered plot based on its wavelength and intensity. Six clusters, each representing droplets having the corresponding fluorescence wavelength and intensity were shown. The TERT and 815A clusters showed the fluorescence of the VIC dye at two distinct intensities; the 815G clusters showed the fluorescence of the FAM dye; and the RNaseP and E1a clusters showed the fluorescence of both the FAM and the VIC dye at distinct intensities (Figure 6). The number of droplets, each having one or more sequences selected from TERT, RNaseP, E1a, 815A and 815G, can be determined from the scattered plot. The copy number of RNaseP, E1a, 815A and 815G in the template were determined by the ratio between the number of droplets having the RNaseP, E1a, 815A and/or 815G sequences and the number of droplets having the TERT sequence (Figure 6).

In yet another exemplary demonstration of multiplexed primer pairs in a droplet-based digital PCR reaction, two droplet libraries were generated: droplet library A was generated where each droplet contained only one primer pair; and droplet library B was generated where the primer pairs were multiplexed at 5x level in each droplet. HapMap sample NA18858 was processed in duplicate with droplet libraries A or B using standard procedures. Two μg sample DNA was used for droplet library A and one μg sample DNA was used for the 5x multiplex droplet library B. After PCR amplification, both droplet libraries were broken and purified over a Qiagen MinElute column and then run on an Agilent Bioanalyzer. Samples were sequenced by Illumina on the Illumina GAII with 50 nucleotide reads and the sequencing results were analyzed using the standard sequencing metrics. The results from the 5x multiplexed droplet library B

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were compared to the singleplex droplet library A using standard metrics shown in the Table below.

The results obtained from the 5x multiplexed droplet library B were equivalent or better than what was obtained from droplet library A. The multiplexing of primers delivers the same sequencing results for base coverage, specificity and uniformity that the singleplexing does with the added advantage of reduced input DNA.

Sample	Total reads	Mapped reads	Specificity	Mean base coverage	C1	C20	C100	Base coverage (0.2x of mean)
Library A with sample 1	27431697	99.4%	0.813	1394	99.5%	99.0%	98.2%	92.8%
Library A with sample 2	15147288	99.4%	0.862	819	99.1%	98.2%	87.6%	78.0%
Library B with sample 1	27861378	99.5%	0.847	1472	99.7%	99.3%	97.6%	89.9%
Library B with sample 2	25758406	99.1%	0.837	1321	99.8%	99.4%	97.9%	91.3%

Total reads: total number of sequencing read found within the provided sample data.

Mapped reads (%): percentage of total reads that mapped to the human genome.

Specificity: percentage of mapped reads that include the target. The target includes all amplicon sequences with primer sequences excluded.

Mean base coverage: average base coverage within the target. The target includes all amplicon sequences with primer sequences excluded.

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C1: % of target that has at least 1x base coverage. Note: non-unique sequencing reads are mapped randomly.

C20: % of target that has at least 20x base coverage.

C100: % of target that has at least 100x base coverage.

Base coverage (0.2x of mean): % of target that has at least 20% of mean base coverage.

Monochromatic Gene Copy Number Assay

Traditional digital PCR methods involve the use of a single labeled probe specific for an individual target. Figure 7 is a schematic depicting one-color detection of a target sequence using droplet based digital PCR. As shown in Panel A of Figure 7, a template DNA is amplified with a forward primer (F1) and a reverse primer (R1). Probe (P1) labeled with a fluorophore of color 1 binds to the target genetic sequence (target 1). Microdroplets are made of diluted solution of template DNA under conditions of limiting or terminal dilution. Droplets containing the target sequence emit fluorescence and are detected by laser (Panels B and C). The number of microcapsules either containing or not containing the target sequence is shown in a histogram (D) and quantified (E).

Figure 8 is a schematic depicting two-color detection of two genetic sequences with a microfluidic device. As shown in Panel A of Figure 8, a template DNA is amplified with two sets of primers: forward primer (F1) and a reverse primer (R1), and forward primer (F2) and a reverse primer (R2). Probe (P1) labeled with a fluorophore of color 1 binds to the target 1 and probe (P2) labeled with a fluorophore of color 2 binds to the target 2 (Panels B and C). Droplets are made of diluted solution of template DNA under conditions of limiting or terminal dilution. Droplets containing the target sequence 1 or 2 emit fluorescence of color 1 or 2 respectively and are optically detected by laser (Panels B and C). The number of microcapsules containing target 1 or 2 is shown by histogram in Panel D.

Methods of the invention involve performing accurate quantitation of multiple different DNA targets by dPCR using probes with the same fluorophore. Figure 9 is a schematic depicting two-color detection of three genetic sequences with a microfluidic device. As shown in Panel A of Figure 9, a template DNA is amplified with three sets of primers: forward primers (F1, F2 and F3) and reverse primers (R1, R2 and R3). Probes (P1, P2 and P3) are labeled with fluorophores (color 1, color 2 and color 1) and bind to the target genetic sequences (target 1,

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target 2 and target 3) (Panels B and C). Microdroplets are made of diluted solution of template DNA under conditions of limiting or terminal dilution. Microdroplets containing target sequence 1 or 3 emit fluorescence of color 1 at two different intensities; and microdroplets containing target sequence 2 emit fluorescence of color 2. The number of microdroplets containing target 1, 2 or 3 is shown by histogram in Panel D.

Recent results from the droplet digital PCR (dPCR) shows that multiple independent PCR reactions can be run and separately quantified using the same fluorophore. Specifically, an SMN2 assay yields an unexpected population of droplets with slightly elevated signal in the FAM detection channel.

The results are depicted in Figure 10. The left-side dot plot in Figure 10 depicts the effect of having the SMN1 blocker present in the reaction. The four clusters depicted in the left-side dot plot are as follows: the top left cluster includes microdroplets containing the reference sequence (SMARCC1); the bottom left cluster includes microdroplets not containing any sequence; the bottom middle cluster includes microdroplets containing sequence for SMN1; and the bottom right cluster includes microdroplets containing sequence for SMN2. The dot plot on the right-side of Figure 10 depicts four clusters where no SMN1 blocker was present in the reaction: the top left cluster includes microdroplets containing the reference sequence (SMARCC1); the bottom left cluster includes microdroplets not containing any sequence; the bottom middle cluster includes microdroplets containing sequence for SMN1; and the bottom right cluster includes microdroplets containing sequence for SMN2. The shift of the bottom middle cluster in right panel as compared to left panel confirms that fluorescence intensity provides a very sensitive measurement for the presence of a sequence.

Without intending to be bound by any theory, the simplest explanation is that the cluster arises from weak association of the SMN2 probe to the SMN1 gene despite the presence of a blocker to that gene (a nonfluorescent complementary probe to the SMN1 gene).

One definitive confirmation of SMN1 as the source of the unexpected cluster was an observed dependence of the intensity of this feature on the presence of the SMN1 blocker. A clear shift toward higher FAM fluorescent intensities was observed in the absence of the blocker (Figure 10). In another definitive confirmation the ratio of the SMN1 (putative) population size to the reference size of 0.96 in perfect agreement with expectation (two copies of each) (S_131

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sample). Another sample, S_122, with the same number of SMN1 copies yielded a ratio of 0.88 in one run and 0.93 in another, also consistent with the proposed explanation of the unexpected cluster.

Without intending to be bound by any theory, these observations indicate that SMN2 probe binding to SMN1 DNA yields an elevated fluorescent signal. A simple kinetic model explaining this phenomenon assumes that the hybridization of the SMN2 probe to the SMN1 DNA achieves equilibrium at a faster rate than the polymerase fills in the complementary strand. The amount of probe fluorophore that is released in each thermal cycle is therefore proportional to (or even equal to) the number of bound probes. Thus the lower the binding affinity the fewer the number of probe fluorophores that are released. Due to SMN1 sequence mismatch(es) with the SMN2 probe, the affinity of the probe is certainly expected to be lower to SMN1 than SMN2. This model also explains the signal dependence on the sMN1 blocker: the blocker competitively inhibits the SMN2 probe hydrolysis by the polymerase exonuclease activity.

It may also be, however, that the probe hybridization does not reach equilibrium before exonuclease activity. In this case, the association rates would play a more dominant role. Similar logic applies. The binding rate to the matching site is likely to be faster than to the mismatch site, and the blocker would act to decelerate probe binding to the mismatch site. The binding of SMN2 probe to SMN1 DNA might be detectable by conventional bulk qPCR, especially in absence of SMN2, but highly quantitative results like those shown here are very unlikely. Definitely, there is no report of qPCR or any other technique quantifying two different DNA sequence motifs with the same color fluorophore. Sequestration of the individual reactions by single molecule amplification within droplets eliminates any confusion regarding mixed contributions to the signal.

The advantage of quantifying DNA with multiple probes of the same color fluorophore extends beyond the example of two highly homologous sequences shown here. Rather, any plurality of sequences of any degree of similarity or dissimilarity can be quantified so long as the different probes have significantly different binding occupancies to their respective DNA binding sites.

Another advantage of the dPCR approach for multiplexed reactions is that the different reactions do not compete with each other for reagents as they would in a bulk qPCR assay.

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However, the possibility for unintended cross-reactivity remains. A multiplexes assay can require a more dilute sample. For instance, at 10% occupancy a duplex reaction would have double occupancy 1% of the time. Hence 1 in 10 PCR+ droplets would be doubles, resulting in a final intensity at least as high and possibly higher than the brighter of the two probes. For a simple duplex system the contribution from each probe could be recovered. In this example the total number of PCR+ droplets for probe 1 would be (Probe 1) + (Probe1+Probe2).
Higher degrees of multiplexing would require greater dilution. For example, for a 4-plex at 1% occupancy the probability of one probe overlapping any of the other 3 is ~3%, and that error may be too high for some applications. The need for large dilutions strongly favors the large number of dPCR reactions.

In another example of the invention, a single fluorophore (FAM) was used in a gene copy number assay for both the reference and the target DNA. A model system was used with varying concentrations of plasmid DNA to represent a change in the target gene copy number, relative to a reference gene, equivalent to 0–16 copies of the target gene per cell. BCKDHA and SMN2 plasmid DNA served as the reference and target with 1× and 0.5× primers and probes respectively. With a starting ratio of 8:1 SMN2 to BCKDHA, the sample was diluted serially by 2× into a solution of BCKDHA at the same concentration to vary just the amount of SMN2. The resultant samples were emulsified, thermally cycled, and over 10^5 droplets were analyzed for each sample as described in the previous section. The process was repeated in triplicate.

Methods of the invention also include analytical techniques for identification of fluorescence signatures unique to each probe. In this example of the invention, histograms of the droplet fluorescence intensities are shown in Fig. 11a for three different template DNA samples: a no template control (dotted line), BCKDHA only (solid line), and 1:1 BCKDHA to SMN2 (dashed line). For clarity, the histograms are shown both overlapped to highlight the similarity for certain peaks, and offset from each other to reveal all of the features. In the case of 1:1 BCKDHA to SMN2, three populations were readily apparent: a dominant feature appeared at 0.08 V, and two smaller peaks were evident at 0.27 and 0.71 V. The dominant feature at 0.08 V was assigned to PCR(–) droplets since both small peaks disappeared, but the large one remained, in the no template control. The peak at 0.71 V was assigned to BCKDHA since it was the sole feature arising with the addition of just BCKDHA, and the peak at 0.27 V appeared on subsequent addition of SMN2, completing the assignments. A very small peak appeared at ~0.9

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V, not visible on the scale of Fig. 11a, that corresponded to droplets occupied by both genes. As another method of the invention, once the different peaks are identified, droplets within each peak were counted corresponding to each possible state (PCR(+) for either BCKDHA or SMN2, or both, or PCR(–)), and the gene copy number was then determined from the ratio of occupancies. Gene copy numbers for each sample in the serial dilution are plotted in Fig. 11b against expected values (observed ratios of SMN2 to BCKDHA to expected ratios of SMN2 to BSKDHA), with an excellent linear fit ($y = 1.01x$) across the full range ($R^2 = 0.9997$, slope = 1.01), demonstrating accurate and precise measurement of the equivalent of 0 to 16 copies of SMN2 per cell.

Detection of alternatively spliced transcripts

The same principle can be used to detect and count alternatively spliced transcripts. TaqMan assays can be designed that are specific for each of the exons in an RNA transcript. After the RNA is turned into cDNA it can be encapsulated into a droplet at 1 copy or less per droplet. The droplet would also contain the multiplexed TaqMan assay for each of the exons. Each of the TaqMan assays would contain a different probe but all the probes would have the same fluorescent dye attached. The droplets would be thermocycled to generate signal for each of the TaqMan assays. If there are multiple splice variants in the sample they each will contain a different number of exons depending on the splicing events. The fluorescent intensity of each droplet would be different depending on the number of exons present. By counting the number of droplets with different intensities it would be possible to identify the presence and abundance of different splice variants in a sample.

Copy number variants in a heterogeneous sample

It would be possible to determine if a heterogeneous sample contained components with different copy level numbers. If the copy number variants to be assayed were spaced close enough along the chromosome, the DNA from a sample could be fragmented and encapsulated in droplets at a level of one haploid genomic equivalent or less per droplet. The droplet would also contain a TaqMan assay specific for the copy number variant. The intensity of the signal in each droplet would depend on the number of copy number variants are present for the sample.

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Counting of the number of droplets of different intensities would indicate things like how many cells in a particular sample had what level of copy number variants.

Tuning TaqMan® Probe Fluorescence Intensity

Identifying probes by fluorescence intensity often requires adjusting the brightness of the probes, particularly for higher-plex assays with dense probe patterns. In the previous section the probes for the gene copy number assay yielded very well resolved peaks (Fig. 11a). Clearly room exists to accommodate one or multiple extra probes in the copy number assay within the resolution of the measurement, but a method for adjusting the fluorescence intensity of the new probes is required to avoid interference with the existing assay. One method of the invention involves varying the probe and primer concentrations together as a very simple technique to optimize relative intensities in higher-plex reactions.

Figure 12 is a schematic for tuning the intensity of a detectable label to a particular target with a microfluidic device. As shown in Panel A of Figure 12, a template DNA is amplified with two sets of primers: forward primers (F1 and F2) and reverse primers (R1 and R2). Probes (P1 and P2) are labeled with fluorophore of color 1 and bind to target 1 and target 2 respectively. Fluorescence from target 2 is lower in intensity than that from target 1 due to single base mismatch between P2 and target 2. As shown in Panel B, template DNA is amplified with two sets of primers: forward primers (F1 and F2) and reverse primers (R1 and R2) (Panel B). Fluorescence from target 2 is lower in intensity than that from target 1 due to the presence of a competing probe 2 that is not labeled with the fluorophore. As shown in Panel C, template DNA is amplified with two sets of primers: forward primers (F1 and F2) and reverse primers (R1 and R2). Probes (P1 and P2) are labeled with fluorophore of color 1 and bind to target 1 and target 2 respectively. Fluorescence from target 2 is lower in intensity than that from target 1 due to the presence of a competing probe 2 that is labeled with a different fluorophore.

Fig. 13 shows probe fluorescence intensities throughout a serial dilution of the probes and primers for a different reference gene, ribonuclease P (RNaseP), against a constant amount of genomic DNA from the Coriell cell line NA3814 at an occupancy of 0.02 target DNA molecules per droplet. The probe fluorescent intensities varied in direct proportion to probe concentration over a narrow concentration range spanning ~0.15 to 0.4 μM ($R^2 = 0.995$)—roughly centered about the typical probe concentration of 0.2 μM—after compensation for dilution errors and

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other run-to-run differences such as optical realignments using the intensity of the PCR(–) droplets as a reference. In summary, probe intensities can be varied by dilution over a small but adequate range for the purpose of tuning multiplexed assays without affecting the amplification itself.

Although the example above for adjusting probe fluorescence intensities involves varying probe and primer concentrations together by the same factor, the invention is not limited to this method alone for varying probe intensity. Other methods known to those familiar with the art for varying probe intensities are also considered. Such methods include varying just the probe concentration; varying just the primer concentrations; varying just the forward primer concentration; varying just the reverse primer concentration; varying the probe, forward, and reverse primers concentrations in any way; varying the thermal cycling program; varying the PCR master mix; incorporating into the assay some fraction of probes that lack fluorophores; or incorporating into the assay any hybridization-based competitive inhibitors to probe binding, such as blocking oligomer nucleotides, peptide nucleic acids, and locked nucleic acids. The invention incorporates the use of these methods adjusting probe fluorescence intensity, or any other methods for adjusting probe fluorescence intensity, used either by themselves or in any combination.

Higher-plex Reactions

One method of the invention involves performing higher-plex assays with a single probe color (i.e. fluorophore). As described above, probe fluorescent intensities can be adjusted by a variety of means such that each intensity level uniquely identifies a DNA target. For example, targets T1, T2, T3, and T4 might be uniquely identified by intensity levels I1, I2, I3, and I4. Not intending to be bound by theory, the maximum number of intensity levels possible for unique identification of targets is related to the resolution of the different intensity levels—that is the spread of intensities for each particular probe compared to the separation between the average intensities of the probes—and it is also related to the intensity of the empty droplets that tends to grow with increasing numbers of probes. The number of intensity levels can be 0, or 1, or 2, or 3, or 4, or up to 10, or up to 20, or up to 50, or up to 100. The number of intensity levels can be higher than 100. In the examples show below, as many as three intensity levels are demonstrated.

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Another method of the invention involves performing higher-plex assays using multiple different probe colors (i.e. fluorophores). As above for the monochromatic multiplexing assay, for each color probe, multiple targets can be identified based on intensity. Additionally, multiple colors that are spectrally separable can be used simultaneously. For example, a single droplet might contain four different probes for measuring four different targets. Two probes might be of color A with different intensities (say, A1 and A2), and the other two probes of color B with different intensities (say B1 and B2). The corresponding targets are T1, T2, T3, and T4 for A1, A2, B1, and B2 respectively. If a droplet shows an increase in fluoresce in color A, the droplet therefore contained either targets T1 or T2. Then, based on the fluorescence intensity of color A, the target could be identified as T1 or the target could be identified as T2. If, however, a droplet shows an increase in fluorescence in color B, the droplet therefore contained either targets T3 or T4. Then, based on the fluorescence intensity of color B, the target could be identified as T3 or the target could be identified as T4. Not intending to be bound by theory, the maximum number of different colors possible is limited by spectral overlap between fluorescence emission of the different fluorophores. The maximum number of colors can be 1, or 2, or 3, or 4, or up to 10, or up to 20. The maximum number of colors can be higher than 20. In the demonstrations that follow, the largest number of colors is two.

Another method of the invention involves performing higher-plex assays using multiple different probe colors (i.e. fluorophores), however unlike the strategy above where each target is identified by single type of probe with a unique color and intensity, instead in this method a single target may be identified by multiple probes that constitute a unique signature of both colors and intensities. For example, a single droplet might contain four different probes for measuring three different targets (say, T1, T2, and T3). Two probes might be of color A (say, A1, and A2), and two probes might be of color B (say, B1 and B2). T1 is measured by probe A1, T2 is measured by probe B1, but T3 is measured by both probes A2 and B2. Thus, when a droplet contains T1 only increased fluorescence appears in color A. When a droplet contains T2 only increased fluorescence appears in color B. However when a droplet contains T3, increased fluorescence appears in both colors A and B.

Generally, without wishing to be constrained by theory, the above three methods for higher-plex dPCR are simplest to implement under conditions of terminal dilution, that is when the probability of multiple different target molecules co-occupying the same droplet is very low

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compared to the probability of any single target occupying a droplet. With multiple occupancy arises the complexity of simultaneous assays competing within the same reaction droplet, and also complexity of assigning the resulting fluorescence intensity that involves a combination of fluorescence from two different reaction products that may or may not be equal to the sum of the two fluorescence intensities of the individual reaction products. However, methods of the invention can accommodate these complications arising from multiple occupancy.

Methods of the invention for higher-plex reactions also include methods for primer and probe pairing. In the simplest case targets are unlikely to reside on the same DNA fragments, such as when targets are from different cells; or when targets are from different chromosomes within a single cell type; or when targets are distant from each other within a single chromosome such that they become physically separated during DNA fragmentation; or when targets are very close to each other within a chromosome, but nevertheless become separated by targeted cleavage of the DNA, such as by restriction enzyme digestion; or for any other reason. In such cases each probe can be paired with a single set of primers (forward and reverse). However, in other cases the target regions might frequently reside on the same DNA fragments, for example when targets reside within the same codon, or for any other reason. In such cases , a single set of primers might serve for multiple probes (for an example, see Pekin *et al.*).

Higher multiplex reactions can be performed to distinguish the haplotypes of two SNPs. For example, assume that at position one there can be genotypes A or A' and at position two there can be genotypes of B or B'. In a diploid genome four unique haplotypes are possible (A,B; A,B';A',B; and A',B'). If for example A' and B' represent drug resistant mutations for infection, it is often the case that A'B and AB' are less sever and treated differently than A'B' which represents a significant drug resistance that must be treated with extreme care. Digital PCR with intensity discrimination is ideally suited for identifying low prevalence of A'B' in a background of mixtures of the other three haplotypes. Haplotyping information is also important for construction of haplotypes in HLA. One way that the present example can be constructed is by assay design such that color one is used for A and is of high or low intensity indicative of allele A or A' respectively and color two is used for B and is of high or low intensity respectively indicative of B or B'. Populations of [color1,color2] corresponding to [Low, Low] would be a measure of an allele of AB and [high, low] allele A'B and an allele of [A'B'] will be readily distinguishable as [high, high] even in a background that is predominately a mixture of

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A'B and AB'. See Fig. 22. In some cases it will be advantageous to start by encapsulating into the droplets long single molecules of nucleic acid that contain both A and B SNP location and in other cases it will be desirable to start by encapsulating single cells, bacteria or other oragnism within the droplets prior to releasing the nucleic acid from the organism. In still other embodiments the multiplex intensity detection of multiple simultaneous targets can be used as surrogate markers for multiple types of binding interactions or labeling of target materials. This technique is also not limited to single molecule detection and can be used for haplotype detection in single cells (e.g., bacteria, somatic cells, etc.). In single cell analysis, a sorting step may be applied prior to haplotyping.

5-plex Assay for Spinal Muscular Atrophy

An aspect of the invention was reduced to practice in an example demonstration of the quantitation of several genetic markers for spinal muscular atrophy (SMA). SMA was selected for one of the example demonstrations due to both its important clinical significance as well as its complicated genetics. It is the second-most prevalent fatal neurodegenerative disease and affects ~1 in 10,000 live births. SMA is most often caused by homozygous absence of exon 7 within the survival of motor neuron 1 gene (SMN1, reviewed by Wirth *et al.*), however the severity of the condition is modulated by the number of gene copies of SMN2 with prognosis ranging from lethal to asymptomatic over 1–5 copy numbers (reviewed by Elsheikh *et al.*). Hence accurate quantitation of SMN2 copy number is important for clinical prognosis and genetic counseling. Aside from large deletions of SMN1, a number of single point mutations or short deletions / duplications within the same gene also account for ~4% of cases of SMA. In a significant step toward a comprehensive SMA assay, the multiplexed dPCR assay demonstrated here contains both copy number assays (for SMN1 & 2) and an assay for one of the prevalent SNPs (c.815A>G).

One embodiment of the invention is a 5-plex assay for SMA diagnostics. The 5-plex assay quantifies common genetic variants impacting SMA including two copy number assays for the SMN1 and SMN2 genes with BCKDHA as a reference, and a SNP assay for the c.815A>G mutation. Two differently colored fluorophores, FAM and VIC, were used to uniquely identify each of the assays. The probes for SMN1 and SMN2 contained only FAM, and for c.815A only VIC. However, mixtures of VIC and FAM-labeled probes were used for BCKDHA and c.815G. The use of VIC and FAM fluorophores in this example does not limit the invention, rather the

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5-plex assay can be used with any spectrally separable fluorophores compatible with the TaqMan assay, or any other fluorogenic hybridization-based probe chemistries. For validating the assay, a model chromosome was synthesized containing a single target region for each of the different primer/probe pairs. EcoRV restriction sites flanked each target, allowing separation of the fragments.

As another method of the invention, histogram-based data presentation and analysis is incorporated into the invention for identifying and characterizing statistically similar populations of droplets that arise from one probe signature (color and intensity), and for discriminating one population of droplets from the others. Fig. 14a shows a 2-dimensional histogram of droplet fluorescence intensities as a contoured heat map, with hotter colors representing higher occurrences. Standard techniques were used to compensate for spectral overlap of the FAM and VIC signals. Samples were run at 0.006 occupancy per target. Six populations were clearly evident, five for the assay and one for PCR(–) droplets. As one method of the invention, the populations were assigned by selective exclusion of assay components. For example, excluding the SMN2 primers and probe eliminated the population at the bottom right in the histogram, but otherwise the distribution remained unchanged. Assignments are labeled in Fig. 14a. As we have found to be generally true for this method of multiplexing, the assay worked immediately with well resolved or at least distinguishable populations for each target. As another method of the invention, the relative positions of the different populations in the histogram were then adjusted into a regularly spaced rectangular array by tuning the probe concentration as described in the previous section. Usually no more than two iterations are required for optimization.

In another method of the invention, the different populations were sufficiently well resolved to allow droplets within each population to be counted by integration across rectangular boundaries. The boundaries were positioned at mid-sections between neighboring peaks. The methods of the invention are not constrained to rectangular boundaries, or to specific boundary locations between peaks. Rather, any closed or unclosed boundary condition can suffice. Boundary conditions do not need to be "binary" either, in the sense that weighted integrations can also be performed across the boundaries to arrive at droplet counts. The peak position of each cluster varied by no more than 2% from run to run after normalization to the intensity of the empty droplets to account for variations in detection efficiency (data not shown). Hence, once identified, the same boundaries for integration could be reused between samples. The methods

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of the invention are not limited to fixed boundary positions. Dynamic population identification and boundary selection in between samples or studies is anticipated. Twenty different patient samples from the Coriell cell repositories were analyzed with this assay: 4 afflicted with SMA, 1 SMA carrier, and 15 negative controls. Assay results are shown in Fig. 14b. Gene copy number was calculated as before, as the ratio of occupancies derived from the number of target droplets *vs.* reference droplets. Like the copy number measurement in Fig. 11, each assay yielded ratios very close to the expected integer values, but when all of the patient data was plotted as actual ratio *vs.* expected integer ratio a small systematic deviation from the ideal slope of 1 was observed. Measured slopes were 0.92, 0.92, and 0.99 for SMN1, SMN2, and c.815A respectively. For clarity, the data in Fig. 14b was scaled to the ideal slope of 1.

The measured genotypes of the different patients were consistent with their disease conditions (unafflicted, carrier, or afflicted). The patients afflicted with SMA each had zero copies of SMN1 (numbers SMA 1–4 in Fig. 14b), the carrier had just one copy, and the negative controls all had two or three copies (numbers 1-15). Three unrelated individuals (numbers 6, 8, and 9) had three copies of SMN1, occurring at a rate of 20% which is similar to a previous report for healthy individuals. Variability in SMN1 copy number is not surprising since it lies within an unstable region of chromosome 5q13. A larger variety of SMN2 copy numbers was observed. One to two copies were most common in the control group, although one individual had zero copies, a distribution consistent with expectations for normal individuals. The SMA carrier and afflicted patients had elevated copy numbers of SMN2 on average: 5 for the carrier, two afflicted with 3 copies, and the others with 2 copies. The afflicted patients were all diagnosed as SMA Type I, the most severe form, based on clinical observations according to the Coriell repository. The strong genotype/phenotype correlation between SMN2 copy number and disease severity suggests that the two individuals with three copies of SMN2 might have an improved Type II prognosis, especially for the patient SMA 1 who had survived to three years at the time of sampling, much beyond the typical maximum life expectancy for SMA Type I of 2 years. However there remains reluctance to predict disease outcome based on SMN2 copies alone since other less well characterized or unknown modifying genes may impact prognosis and because not all SMN2 copies may be complete genes. Furthermore some Type I patients have begun surviving longer in newer clinical settings. Hence, with little clinical information regarding the

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patients available to us, we can conclude that our SMN2 assay results were consistent with broad expectations for disease severity.

The SNP assay revealed that all patients carried the normal c.815A genotype and no instances of c.815G were observed. The mutation is relatively rare and hence was not expected to appear in a small patient panel. Of interest, however, was the presence of an apparent extra gene fragment in two unrelated individuals that was uncovered with the SNP assay. The c.815A>G assay does not discriminate between SMN1 and SMN2 due to their high sequence similarity, and hence the total copies of c.815A and G should equal the sum of the copies of SMN1 and SMN2. This was true for all patients except for healthy patients number 1 and 2, both of whom had one extra copy of c.815A. c.815 lies on exon 6, and the SNP that discriminates between the SMN1 and SMN2 genes lies on exon 7, hence the extra genes may be fragments of SMN1 lacking exon 7. This seems reasonable because the deletion of exon 7 is the common mutation causing 95% of cases of SMA (reviewed by Wirth *et al.*) and it is carried by 1/40 to 1/60 adults. Thus these patients might have been typical carriers of SMA but for the acquisition of at least one compensating healthy copy of SMN1 on the same chromosome.

9-Plex Assay for Spinal Muscular Atrophy

A 9-plex assay for certain SMA related targets was also demonstrated with just two colors (probes containing FAM and VIC fluorophores). Aside from the optimized primer and probe concentrations, assay conditions and experimental procedures were identical to the 5-plex assay above. Figure 15a shows the various droplet populations in 2-D histograms before optimization of probe concentrations. The identity of the different targets is shown on the figure itself. As one method of the invention, the identification of the different populations was made as before, by selective exclusion and/or addition of one or more assays. Most of the populations were already well resolved, with the exception of the probe for the c.815A genotype that was in close proximity with the cluster corresponding to empty droplets. After three iterations of optimization of probe concentrations, all of the target populations were well resolved from each other, and well resolved from the empty droplets (Figure 15b). Three methods of the invention were highlighted in this demonstration: (1) nine DNA targets were uniquely identified in a two-dimensional histogram, far beyond the capabilities of conventional qPCR; (2) target DNA molecules were distinguished on the basis of some combination of both color and intensity

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arising from one or multiple probes against the same target; and (3) the relative positions of the target molecules within the histogram were adjusted by varying the probe concentrations to optimize the pattern of colors and intensities for increased resolution amongst the various droplet populations.

As one method of the invention, different droplet populations were identified by selective addition or exclusion of assays in the examples above. However the invention is not limited to this method alone. Rather, any method for population assignments known to those in the art are considered. Methods of the invention include any method that can cause an identifiable displacement, appearance, or disappearance of one or more populations within the histograms including changing the probe and primer concentrations together, either by the same factor or by different factors; changing the probe concentration alone; changing the primer concentrations alone; changing the thermal cycling conditions; and changing the master mix composition. Another method of the invention takes advantage of prior knowledge of the position of an assay within a histogram to assist assignment.

Multiplexing Capacity

The level of multiplexing demonstrated in the preceding SMA example was 9×, significantly exceeding the maximum practicable number with qPCR. Without wishing to be constrained by theory, the two main limitations are the resolution between assays and the increasing fluorescence intensity of empty droplets with higher loading of probes. A method of the invention involves optimizing the pattern of colors and intensities of the different probes for maximum multiplexing while still achieving adequate specificity for each individual reaction. Although rectangular arrays of droplet populations were demonstrated for the 5- and 9-plex reactions, another desirable pattern is the tight-packed hexagonal array. However the invention is not constrained to any particular array strategy.

Adding extra colors would increase the capability even further, however with some diminishing returns because the fluorescence of the empty droplets would continue to rise. The capacity could be yet further increased with better probes yielding larger differential signals, such as hybrid 5'-nuclease / molecular beacon probes that reduce background by contact quenching yet exhibit the bright signals typical of free unquenched fluorophores. With such improvements multiplexing capacity exceeding 50× can be envisioned.

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Combined Multiplexing with Optical Labeling

Using droplet-based microfluidics, multiple targets can also be measured simultaneously by a different method. According to the alternative method, primers and probes can be loaded individually into droplets along with an optical label to uniquely identify the assay. Typically the optical label is a fluorophore, or a combination of different fluorophores, that are spectrally distinct from the probe fluorophore. Various different types of droplets, each containing different assays that are uniquely identified by different optical labels, can be mixed into a "library" of droplets. Then, according to methods of the invention above, library droplets are merged one-to-one with droplets containing template DNA. After thermal cycling, some droplets that contain template DNA will exhibit brighter fluorescence at the emission wavelengths of the probes. The specific target DNA molecules giving rise to these PCR(+) signals are subsequently identified by the optical probes. In one study, the six common mutations in *KRAS* codon 12 were screened in parallel in a single experiment by one-to-one fusion of droplets containing genomic DNA with any one of seven different types of droplets (a seven-member library), each containing a TaqMan® probe specific for a different *KRAS* mutation, or wild-type *KRAS*, and an optical code.

In one method of the invention, optical labeling can be combined with the various methods for multiplexing dPCR already incorporated into this invention. For example, a single optical label might code for the entire 5-plex SMA assay, above, instead of just a single assay as in the *KRAS* example above. In this manner, other optical labels might code for different screening assays for newborn infants. According to other methods of the invention, above, a single DNA sample from an infant could then be analyzed with all of the assays simultaneously by merging droplets containing the DNA one-to-one with library droplets containing the optically encoded assays.

As an example of combining multiplexing with optical labels, a so called 3x3x3 combination multiplex reaction with optical labeling was demonstrated (3x3 optical labeling with two fluorophores, each encoding a triplex assay, for a total of 27-plex). Two fluorophores were employed for optical labeling, Alexa633 and CF680 (excited by a 640 nm laser), with three intensity levels each producing nine total optical labels. As before with the 5- and 9-plex assays for SMA, TaqMan assays were used with FAM and VIC fluorophores (excited by a 488 nm laser). The fluorescence from the FAM and VIC fluorophores were recorded simultaneously

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with the fluorescence from the optical labels, requiring modifications to the optical layout of the instrumentation described for the SMA assay (the optical schematic for two-laser excitation and 4-color detection is shown in entirety in Figure 16). Also, co-flow microfluidics were used in this example (the use of co-flow based microfluidics for this application is one of the methods of the invention described above). In this case, the template DNA was introduced into the chip in one flow, and the PCR master mix, the primers and probes for one triplex assay, and the unique composition of fluorophores for the optical label were introduced into the chip in another flow simultaneously. The two flow streams converged in a fluidic intersection upstream from the droplet forming module, and thus each droplet formed contained the contents of both flow streams. Methods to implement co-flow microfluidics are well known to those in the art. The droplets were collected, and then the procedure was repeated with the next triplex assay and optical label. The procedure was repeated a total of nine times, once for each pair of assays and optical labels. All of the droplets were collected into a single PCR tube and thermally cycled off chip. The mixture of thermally cycled droplets was reinjected into the same read-out chip as used for the SMA assay, above, and the fluorescence intensities of the assays from all four fluorophores was recorded.

Figure 17 shows the cumulative results from all droplets in the 3x3x3 assay using co-flow microfluidics. The figure shows two 2-D histograms of droplet fluorescence intensities, the histogram on the left from all of the optical labels, and the histogram on the right from the assays. Standard methods were used to compensate for spectral overlap. The histograms are shown as a heat maps, with hotter colors designating larger numbers of droplets. Nine different clusters of droplets were clearly evident in the histogram of the optical labels, corresponding to each of the nine different optical labels: there is a small group of four clusters at the bottom left corner of the histogram, corresponding to optical labels with the lowest fluorescent intensities; and there are five clusters appearing as linear streaks at the higher intensities. The droplet clusters were less distinct in the histogram for the assay, but this was as expected because the droplets shown contained all of the triplex assays. The individual assays became clearly distinct once a single type of assay was selected by using the optical labels, as follows.

Methods of the invention involve selecting individual populations of droplets all containing the same optical labels, or groups of optical labels. In some methods of the invention, boundaries of fluorescence intensity were used to specify populations. In the example shown

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here, a rectangular boundary was used specifying the minimum and maximum fluorescence intensities for each fluorophore. However the methods of the invention are not restricted to rectangular boundaries. Any boundary, closed or unclosed, can be employed. Furthermore, according to methods of the invention, selections of droplet populations can be made by any method, and is not restricted to threshold-based methods such as boundary selection.

Figure 18A shows the droplet fluorescence intensities for the assay (right histogram) when only one optical label was selected (left histogram). The lines overlaid on the histogram of the optical labels identify the rectangular boundary used to select just the optical label with the lowest fluorescence for both fluorophores. Both histograms showed only the droplets that were selected. After selection, four distinct clusters of droplets appeared in the assay histogram, three for the different assays (in this case, assays for SMN1, SMN2, and TERT, where TERT is another common reference gene) and one for the empty droplets. The copy numbers for SMN1 and SMN2 were measured by the same methods of the invention as described above for the 5-plex SMA assay, with values of 1.8 and 0.94 close to the expected values of 2 and 1, respectively. The same assay was encoded with two other optical labels, and their selections are shown in Figures 18B and C. Similar results were achieved, with an overall measurement of 1.9±0.1 and 0.9±0.1 copies of SMN1 and SMN2 respectively, showing the measurement to be accurate within experimental uncertainty.

Figures 19A, B, and C show optical label selections for a different assay (TERT, c.5C in the SMN1 gene, and BCKDHA (labeled E1a in the figure)). In each case four distinct clusters also appeared, and by the same methods of the invention above, accurate measurements of gene copy number were made for c.5C and BCKDHA, referenced to TERT, of 2.9±0.1 and 2.0±0.2 compared to 3 and 2, respectively. Figures 20A, B, and C show optical label selections for a third assay (TERT, c.88G in the SMN1 gene, and RNaseP, where RNaseP is a common reference gene). Accurate gene copy numbers of 2.1±0.1 were measured for both c.88G and RNaseP, referenced to TERT, compared to the expected value of 2.

In summary, the demonstration here shows use of nine different optical labels to enable independent measurement of three triplex assays in a single experiment. Although some of the optical labels encoded for redundant assays in this example (there were only three different assays despite having nine optical labels), the invention is not constrained to any particular formatting of assays and optical labels. Embodiments of the invention include formats where all

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of the assays are the same across all of the optical labels; where none of the assays are the same across all of the optical labels; where some of the assays are the same across all of the optical labels; where some of the assays have greater plexity than others across all of the optical labels; where all of the assays have the same plexity across all of the optical labels; and any other arrangements of assays across all of the optical labels are considered.

Although two different fluorophores were used to create the optical labels in this example, the invention is not constrained to any particular number of fluorophores comprising the optical labels. Embodiments of the invention include optical labels comprised of 1 fluorophore, or 2 fluorophores, or 3 fluorophores, or 4 fluorophores, or up to 10 fluorophores, or up to 20 fluorophores. Optical labels can also comprise more than 20 fluorophores.

Although solely triplex assays were used in the example demonstration here, the invention is not constrained to use of triplex assays with optical labels. Embodiments of the invention include plexities of the following amounts when used with optical labels: single plex, duplex, triplex, 4-plex, up to 10-plex, up to 20-plex, up to 50-plex, and up to 100-plex. Embodiments of the invention also include plexities exceeding 100 when used with optical labels.

Another method of the invention involves the use of droplet merging, instead of co-flow, for combining multiplexing with optical labels. A demonstration using droplet merging was performed with the same 3x3x3 assay as in the preceding example with co-flow. The assays (probes and primers) combined with their unique optical labels were first encapsulated into droplets along with the PCR master mix. Subsequently, according to methods of the invention described above, a library containing a mixture of droplets from all nine optically labeled assays was merged one-to-one with droplets containing template DNA from the same patient as in the preceding example. As another method of the invention, the droplet merge was performed using a lambda-injector style merge module, as described in U.S. Provisional Application, Serial NO. 61/441,985, incorporated by reference herein. Aside from the differences between co-flow and merge, the assays and experimental procedures were identical to those above for the co-flow experiment. Figure 21 shows 2-D histograms of droplet fluorescence intensity for the optical labels and the assays that are similar to those in Figures 17–20. As in the case for co-flow, upon selection of droplets containing individual optical labels, the expected distinct clusters of droplets corresponding to each assay were clearly evident. Furthermore for each assay the

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measured gene copy number matched or very nearly matched the expected values within experimental uncertainty (See Table 1).

Gene or genotype	*Measured copy number*	*Expected copy number*
SMN1	1.98 ± 0.09	2
SMN2	0.99 ± 0.04	1
c.5C in SMN1	3.01 ± 0.06	3
c.88G in SMN1	2.15 ± 0.08	2
BCKDHA	2.00 ± 0.05	2
RNaseP	2.11 ± 0.16	2

Table 1 Gene copy number measurements from the 3x3x3 assay.

Although methods of the invention include using either microfluidics with co-flow or droplet merging, the invention is not limited in this regard. Any fluidic method capable of generating optically labeled droplets that also contain fluorogenic DNA hybridization probes are considered. For example, other embodiments well known in the art are mixing optical labels and assays in the macrofluidic environment before injection into a droplet generating chip; and mixing optical labels and assays thoroughly upstream from the droplet forming module in dedicated mixing modules, such as with a serpentine mixer.

Data Analysis

One method of the invention involves histogram-based data presentation and analysis for identifying and characterizing populations of statistically similar droplets that arise from unique probe signatures (color and intensity), and for discriminating one population of droplets from the others. Another method of the invention involves histogram-based data presentation and analysis for identifying and selecting populations of droplets based on unique signatures from optical labels. Examples of one and two-dimensional histograms have been provided for these methods, but the invention is not limited in this regard. As described above, it is anticipated that greater numbers of colors will be used for both multiplexing and for optical labels. Hence, embodiments of the invention include histograms of dimensionality greater than two, such as 3,

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or 4, or up to 10, or up to 20. Histograms of dimensionality greater than 20 are also incorporated into the invention.

Another method of the invention involves the selection of droplets within histograms, either for counting, or for assay selection as in the use of optical labels, or for any other purpose. Methods of the invention include selections by boundaries, either closed or unclosed, of any possible shape and dimension. Methods of the invention also include selections of droplets that exhibit fluorescence from single types of fluorophores, or from multiple types of fluorophores, such as arising from multiple probes against a common DNA target.

Polymerase Error Correction

For applications requiring very high sensitivity, such as searching for rare mutations amidst an abundance of wild-type DNA, false positive results can arise from errors from the DNA polymerase itself. For example, during one of the early thermal cycles the polymerase might synthesize the mutant strand of DNA from a wild-type template. This type of error is most likely to occur when the difference between the mutant and the wild-type is very small, such as single nucleotide polymorphism (SNP). In this method of the invention, each droplet contains only a single target nucleic acid, if any at all. In the preferred embodiment, this is accomplished under the conditions of terminal dilution. Droplets that contain amplification products that are a wild-type of the target are detected based on emission from the fluorophore that is released from the probe that hybridizes to the wild-type of the target. Droplets that contain the variant of the target are detected based on emission from the fluorophore that is released from the probe that hybridizes to the variant of the target. Since each droplet starts with only a single nucleic acid molecule, the resultant amplification products in each droplet are either homogeneous for the target or homogenous for the variant of the target.

However, certain droplets will contain a heterogeneous mixture of both target and target variant due to polymerase errors during the PCR reaction. Error rates in PCR vary according to the precise nucleic acid sequence, the thermostable enzyme used, and the in vitro conditions of DNA synthesis. For example, the error frequency (mutations per nucleotide per cycle) during PCR catalyzed by the thermostable *Thermus aquaticus* (*Taq*) DNA polymerase vary more than 10-fold, from -2×10^{-4} to $<1 \times 10^{-5}$. Eckert et al. (Genome Res. 1:17-24, 1991), the content of which is incorporated by reference herein in its entirety. Polymerase-mediated errors at a

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frequency of 1 mutation per 10,000 nucleotides per cycle are an important consideration for any PCR application that begins with a small amount of starting material (e.g., less than a total of 10,000 nucleotides of target DNA) or that focuses on individual DNA molecules in the final PCR population.

The proportion of DNA molecules that contain sequence changes is a function of the error rate per nucleotide per cycle, the number of amplification cycles and the starting population size. The population of altered DNA molecules arises during PCR from two sources: (1) new errors at each PCR cycle; and (2) amplification of DNA molecules containing errors from previous cycles. The formula $f = np/2$ describes the average mutation frequency (f) for PCR amplification as a function of the polymerase error rate per nucleotide per cycle (p) and the number of cycles (n), assuming that p is constant at each cycle. Due to the exponential nature of PCR, the occurrence of an early error can increase the final error frequency above the average described by $f = np/2$, because the variant DNA molecule will be amplified with each cycle, resulting in populations with a larger than average number of variants.

A polymerase error that converts a wild-type of the target to a variant of the target during an early round of amplification results in a heterogeneous population of target and target variant in a droplet, and may lead to a droplet being incorrectly identified as containing a variant of the target, i.e., a false positive. Such false positives greatly impact the validity and precision of digital PCR results.

Methods of the invention are able to detect which droplets contain a heterogeneous population of molecules and are able to exclude those droplets from analysis. As droplets containing amplified product flow in a channel through the detector module, the module is able to detect the fluorescent emission in each droplet. Droplets that produce only a single signal are classified as droplets that contain a homogeneous population of target. Since probes that hybridize to the wild-type of the target have a different fluorophore attached than probes that hybridize to a variant of the wild-type of the target, methods of the invention can classify each droplet as containing either a homogeneous population of amplicons of the target or a homogeneous population of amplicons of the variant of the target.

Droplets that produce two signals are classified as droplets that contain a heterogeneous population of molecules. Since each droplet started with at most a single target nucleic acid, a droplet that includes amplification products that are both amplicons of the target and amplicons

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of a variant of the target are droplets in which the variant of the target was produced by a polymerase error during the PCR reaction, most likely a polymerase error during an early cycle of the PCR reaction. Such droplets are detected and excluded from analysis.

Analysis

Analyze is then performed on only the droplets that contain a homogeneous population of molecules. The analysis may be based on counting, i.e., determining a number of droplets that contain only wild-type target, and determining a number of droplets that contain only a variant of the target. Such methods are well known in the art. See, e.g., Lapidus et al. (U.S. patent numbers 5,670,325 and 5,928,870) and Shuber et al. (U.S. patent number 6,203,993 and 6,214,558), the content of each of which is incorporated by reference herein in its entirety.

Generally, the presence of droplets containing only variant is indicative of a disease, such as cancer. In certain embodiments, the variant is an allelic variant, such as an insertion, deletion, substitution, translocation, or single nucleotide polymorphism (SNP).

Biomarkers that are associated with cancer are known in the art. Biomarkers associated with development of breast cancer are shown in Erlander et al. (US 7,504,214), Dai et al. (US 7,514, 209 and 7,171,311), Baker et al. (US 7,056,674 and US 7,081,340), Erlander et al. (US 2009/0092973). The contents of the patent application and each of these patents are incorporated by reference herein in their entirety. Biomarkers associated with development of cervical cancer are shown in Patel (US 7,300,765), Pardee et al. (US 7,153,700), Kim (US 6,905,844), Roberts et al. (US 6,316,208), Schlegel (US 2008/0113340), Kwok et al. (US 2008/0044828), Fisher et al. (US 2005/0260566), Sastry et al. (US 2005/0048467), Lai (US 2008/0311570) and Van Der Zee et al. (US 2009/0023137). Biomarkers associated with development of vaginal cancer are shown in Giordano (US 5,840,506), Kruk (US 2008/0009005), Hellman et al. (Br J Cancer. 100(8):1303-1314, 2009). Biomarkers associated with development of brain cancers (e.g., glioma, cerebellum, medulloblastoma, astrocytoma, ependymoma, glioblastoma) are shown in D'Andrea (US 2009/0081237), Murphy et al. (US 2006/0269558), Gibson et al. (US 2006/0281089), and Zetter et al. (US 2006/0160762). Biomarkers associated with development of renal cancer are shown in Patel (US 7,300,765), Soyupak et al. (US 7,482,129), Sahin et al. (US 7,527,933), Price et al. (US 7,229,770), Raitano (US 7,507,541), and Becker et al. (US 2007/0292869). Biomarkers associated with development of hepatic cancers (e.g., hepatocellular

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carcinoma) are shown in Horne et al. (US 6,974,667), Yuan et al. (US 6,897,018), Hanausek-Walaszek et al. (US 5,310,653), and Liew et al. (US 2005/0152908). Biomarkers associated with development of gastric, gastrointestinal, and/or esophageal cancers are shown in Chang et al. (US 7,507,532), Bae et al. (US 7,368,255), Muramatsu et al. (US 7,090,983), Sahin et al. (US 7,527,933), Chow et al. (US 2008/0138806), Waldman et al. (US 2005/0100895), Goldenring (US 2008/0057514), An et al. (US 2007/0259368), Guilford et al. (US 2007/0184439), Wirtz et al. (US 2004/0018525), Filella et al. (Acta Oncol. 33(7):747-751, 1994), Waldman et al. (US 6,767,704), and Lipkin et al. (Cancer Research, 48:235-245, 1988). Biomarkers associated with development of ovarian cancer are shown in Podust et al. (US 7,510,842), Wang (US 7,348,142), O'Brien et al. (US 7,291,462, 6,942,978, 6,316,213, 6,294,344, and 6,268,165), Ganetta (US 7,078,180), Malinowski et al. (US 2009/0087849), Beyer et al. (US 2009/0081685), Fischer et al. (US 2009/0075307), Mansfield et al. (US 2009/0004687), Livingston et al. (US 2008/0286199), Farias-Eisner et al. (US 2008/0038754), Ahmed et al. (US 2007/0053896), Giordano (US 5,840,506), and Tchagang et al. (Mol Cancer Ther, 7:27-37, 2008). Biomarkers associated with development of head-and-neck and thyroid cancers are shown in Sidransky et al. (US 7,378,233), Skolnick et al. (US 5,989,815), Budiman et al. (US 2009/0075265), Hasina et al. (Cancer Research, 63:555-559, 2003), Kebebew et al. (US 2008/0280302), and Ralhan (Mol Cell Proteomics, 7(6):1162-1173, 2008). The contents of each of the articles, patents, and patent applications are incorporated by reference herein in their entirety. Biomarkers associated with development of colorectal cancers are shown in Raitano et al. (US 7,507,541), Reinhard et al. (US 7,501,244), Waldman et al. (US 7,479,376); Schleyer et al. (US 7,198,899); Reed (US 7,163,801), Robbins et al. (US 7,022,472), Mack et al. (US 6,682,890), Tabiti et al. (US 5,888,746), Budiman et al. (US 2009/0098542), Karl (US 2009/0075311), Arjol et al. (US 2008/0286801), Lee et al. (US 2008/0206756), Mori et al. (US 2008/0081333), Wang et al. (US 2008/0058432), Belacel et al. (US 2008/0050723), Stedronsky et al. (US 2008/0020940), An et al. (US 2006/0234254), Eveleigh et al. (US 2004/0146921), and Yeatman et al. (US 2006/0195269). Biomarkers associated with development of prostate cancer are shown in Sidransky (US 7,524,633), Platica (US 7,510,707), Salceda et al. (US 7,432,064 and US 7,364,862), Siegler et al. (US 7,361,474), Wang (US 7,348,142), Ali et al. (US 7,326,529), Price et al. (US 7,229,770), O'Brien et al. (US 7,291,462), Golub et al. (US 6,949,342), Ogden et al. (US 6,841,350), An et al. (US 6,171,796), Bergan et al. (US 2009/0124569), Bhowmick (US

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2009/0017463), Srivastava et al. (US 2008/0269157), Chinnaiyan et al. (US 2008/0222741), Thaxton et al. (US 2008/0181850), Dahary et al. (US 2008/0014590), Diamandis et al. (US 2006/0269971), Rubin et al. (US 2006/0234259), Einstein et al. (US 2006/0115821), Paris et al. (US 2006/0110759), Condon-Cardo (US 2004/0053247), and Ritchie et al. (US 2009/0127454). Biomarkers associated with development of pancreatic cancer are shown in Sahin et al. (US 7,527,933), Rataino et al. (US 7,507,541), Schleyer et al. (US 7,476,506), Domon et al. (US 7,473,531), McCaffey et al. (US 7,358,231), Price et al. (US 7,229,770), Chan et al. (US 2005/0095611), Mitchl et al. (US 2006/0258841), and Faca et al. (PLoS Med 5(6):e123, 2008). Biomarkers associated with development of lung cancer are shown in Sahin et al. (US 7,527,933), Hutteman (US 7,473,530), Bae et al. (US 7,368,255), Wang (US 7,348,142), Nacht et al. (US 7,332,590), Gure et al. (US 7,314,721), Patel (US 7,300,765), Price et al. (US 7,229,770), O'Brien et al. (US 7,291,462 and US 6,316,213), Muramatsu et al. (US 7,090,983), Carson et al. (US 6,576,420), Giordano (US 5,840,506), Guo (US 2009/0062144), Tsao et al. (US 2008/0176236), Nakamura et al. (US 2008/0050378), Raponi et al. (US 2006/0252057), Yip et al. (US 2006/0223127), Pollock et al. (US 2006/0046257), Moon et al. (US 2003/0224509), and Budiman et al. (US 2009/0098543). Biomarkers associated with development of skin cancer (e.g., basal cell carcinoma, squamous cell carcinoma, and melanoma) are shown in Roberts et al. (US 6,316,208), Polsky (US 7,442,507), Price et al. (US 7,229,770), Genetta (US 7,078,180), Carson et al. (US 6,576,420), Moses et al. (US 2008/0286811), Moses et al. (US 2008/0268473), Dooley et al. (US 2003/0232356), Chang et al. (US 2008/0274908), Alani et al. (US 2008/0118462), Wang (US 2007/0154889), and Zetter et al. (US 2008/0064047). Biomarkers associated with development of multiple myeloma are shown in Coignet (US 7,449,303), Shaughnessy et al. (US 7,308,364), Seshi (US 7,049,072), and Shaughnessy et al. (US 2008/0293578, US 2008/0234139, and US 2008/0234138). Biomarkers associated with development of leukemia are shown in Ando et al. (US 7,479,371), Coignet (US 7,479,370 and US 7,449,303), Davi et al. (US 7,416,851), Chiorazzi (US 7,316,906), Seshi (US 7,049,072), Van Baren et al. (US 6,130,052), Taniguchi (US 5,643,729), Insel et al. (US 2009/0131353), and Van Bockstaele et al. (Blood Rev. 23(1):25-47, 2009). Biomarkers associated with development of lymphoma are shown in Ando et al. (US 7,479,371), Levy et al. (US 7,332,280), and Arnold (US 5,858,655). Biomarkers associated with development of bladder cancer are shown in Price et al. (US 7,229,770), Orntoft (US 6,936,417), Haak-Frendscho et al. (US 6,008,003), Feinstein

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et al. (US 6,998,232), Elting et al. (US 2008/0311604), and Wewer et al. (2009/0029372). The content of each of the above references is incorporated by reference herein in its entirety.

In certain embodiments, methods of the invention may be used to monitor a patient for recurrence of a cancer. Since the patient has already been treated for the cancer, the genetic profile and particular mutation(s) associated with that patient's cancer are already known. Probes may be designed that specifically hybridize to the region of the nucleic acid that contains the mutation(s) that is indicative of the cancer for which the patient was previously treated. A patient's sample (e.g., pus, sputum, semen, urine, blood, saliva, stool, or cerebrospinal fluid) may then be analyzed as described above to determine whether the mutant allele(s) is detected in the sample, the presence of which being indicative of recurrence of the cancer.

Droplet Sorting

Methods of the invention may further include sorting the droplets based upon whether the droplets contain a homogeneous population of molecules or a heterogeneous population of molecules. A sorting module may be a junction of a channel where the flow of droplets can change direction to enter one or more other channels, e.g., a branch channel, depending on a signal received in connection with a droplet interrogation in the detection module. Typically, a sorting module is monitored and/or under the control of the detection module, and therefore a sorting module may correspond to the detection module. The sorting region is in communication with and is influenced by one or more sorting apparatuses.

A sorting apparatus includes techniques or control systems, e.g., dielectric, electric, electro-osmotic, (micro-) valve, etc. A control system can employ a variety of sorting techniques to change or direct the flow of molecules, cells, small molecules or particles into a predetermined branch channel. A branch channel is a channel that is in communication with a sorting region and a main channel. The main channel can communicate with two or more branch channels at the sorting module or branch point, forming, for example, a T-shape or a Y-shape. Other shapes and channel geometries may be used as desired. Typically, a branch channel receives droplets of interest as detected by the detection module and sorted at the sorting module. A branch channel can have an outlet module and/or terminate with a well or reservoir to allow collection or disposal (collection module or waste module, respectively) of the molecules, cells, small

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molecules or particles. Alternatively, a branch channel may be in communication with other channels to permit additional sorting.

A characteristic of a fluidic droplet may be sensed and/or determined in some fashion, for example, as described herein (e.g., fluorescence of the fluidic droplet may be determined), and, in response, an electric field may be applied or removed from the fluidic droplet to direct the fluidic droplet to a particular region (e.g. a channel). In certain embodiments, a fluidic droplet is sorted or steered by inducing a dipole in the uncharged fluidic droplet (which may be initially charged or uncharged), and sorting or steering the droplet using an applied electric field. The electric field may be an AC field, a DC field, etc. For example, a channel containing fluidic droplets and carrier fluid, divides into first and second channels at a branch point. Generally, the fluidic droplet is uncharged. After the branch point, a first electrode is positioned near the first channel, and a second electrode is positioned near the second channel. A third electrode is positioned near the branch point of the first and second channels. A dipole is then induced in the fluidic droplet using a combination of the electrodes. The combination of electrodes used determines which channel will receive the flowing droplet. Thus, by applying the proper electric field, the droplets can be directed to either the first or second channel as desired. Further description of droplet sorting is shown for example in Link et al. (U.S. patent application numbers 2008/0014589, 2008/0003142, and 2010/0137163) and European publication number EP2047910 to Raindance Technologies Inc.

Based upon the detected signal at the detection module, droplets containing a heterogeneous population of molecules are sorted away from droplets that contain a homogeneous population of molecules. Droplets may be further sorted to separate droplets that contain a homogeneous population of amplicons of the target from droplets that contain a homogeneous population of amplicons of the variant of the target.

Release of Target from Droplet

Methods of the invention may further involve releasing amplified target molecules from the droplets for further analysis. Methods of releasing amplified target molecules from the droplets are shown in for example in Link et al. (U.S. patent application numbers 2008/0014589, 2008/0003142, and 2010/0137163) and European publication number EP2047910 to RainDance Technologies Inc.

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In certain embodiments, sample droplets are allowed to cream to the top of the carrier fluid. By way of non-limiting example, the carrier fluid can include a perfluorocarbon oil that can have one or more stabilizing surfactants. The droplet rises to the top or separates from the carrier fluid by virtue of the density of the carrier fluid being greater than that of the aqueous phase that makes up the droplet. For example, the perfluorocarbon oil used in one embodiment of the methods of the invention is 1.8, compared to the density of the aqueous phase of the droplet, which is 1.0.

The creamed liquids are then placed onto a second carrier fluid which contains a de-stabilizing surfactant, such as a perfluorinated alcohol (e.g. 1H,1H,2H,2H-Perfluoro-1-octanol). The second carrier fluid can also be a perfluorocarbon oil. Upon mixing, the aqueous droplets begins to coalesce, and coalescence is completed by brief centrifugation at low speed (e.g., 1 minute at 2000 rpm in a microcentrifuge). The coalesced aqueous phase can now be removed and the further analyzed.

The released amplified material can also be subjected to further amplification by the use tailed primers and secondary PCR primers. In this embodiment the primers in the droplet contain an additional sequence or tail added onto the 5' end of the sequence specific portion of the primer. The sequences for the tailed regions are the same for each primer pair and are incorporated onto the 5' portion of the amplicons during PCR cycling. Once the amplicons are removed from the droplets, another set of PCR primers that can hybridize to the tail regions of the amplicons can be used to amplify the products through additional rounds of PCR. The secondary primers can exactly match the tailed region in length and sequence or can themselves contain additional sequence at the 5' ends of the tail portion of the primer. During the secondary PCR cycling these additional regions also become incorporated into the amplicons. These additional sequences can include, but are not limited to adaptor regions utilized by sequencing platforms for library preparation and sequencing, sequences used as a barcoding function for the identification of samples multiplexed into the same reaction. molecules for the separation of amplicons from the rest of the reaction materials such as biotin, digoxin, peptides, or antibodies and molecules such as fluorescent markers that can be used to identify the fragments.

In certain embodiments, the amplified target molecules are sequenced. In a particular embodiment, the sequencing is single-molecule sequencing-by-synthesis. Single-molecule sequencing is shown for example in Lapidus et al. (U.S. patent number 7,169,560), Quake et al.

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(U.S. patent number 6,818,395), Harris (U.S. patent number 7,282,337), Quake et al. (U.S. patent application number 2002/0164629), and Braslavsky, et al., PNAS (USA), 100: 3960-3964 (2003), the contents of each of these references is incorporated by reference herein in its entirety.

Briefly, a single-stranded nucleic acid (e.g., DNA or cDNA) is hybridized to oligonucleotides attached to a surface of a flow cell. The single-stranded nucleic acids may be captured by methods known in the art, such as those shown in Lapidus (U.S. patent number 7,666,593). The oligonucleotides may be covalently attached to the surface or various attachments other than covalent linking as known to those of ordinary skill in the art may be employed. Moreover, the attachment may be indirect, e.g., via the polymerases of the invention directly or indirectly attached to the surface. The surface may be planar or otherwise, and/or may be porous or non-porous, or any other type of surface known to those of ordinary skill to be suitable for attachment. The nucleic acid is then sequenced by imaging the polymerase-mediated addition of fluorescently-labeled nucleotides incorporated into the growing strand surface oligonucleotide, at single molecule resolution.

Experimental Detail

What follows is experimental detail for the various experiments details above.

Primers and probes

All TaqMan® primers and probes used here are listed in Table 2. Unless otherwise noted by reference in the table, the primers and probes were designed with the "Custom TaqMan® Assay Design Tool" from Applied Biosystems Inc. (ABI) and procured through ABI (Carlsbad, CA). Probes were labeled with 6-carboxyfluorescein (FAM, λ_{ex} 494 nm \ λ_{em} 494 nm) or VIC™ (from ABI, λ_{ex} 538 nm \ λ_{em} 554 nm).

Target	***Assay***	***Primers (5' to 3')***	***Probe (5' to 3')***	***5-plex assay conditions***	***Ref.***
SMN1	Copy number	(f) AATGCTTTTTAA-CATCCATATAAAGCT (r) CCTTAATTTAAG-GAATGTGAGCACC	FAM–CAGGGTTTC*AGACAAA–MGBNFQ	0.37×	Anhuf *et al.*, 2003
SMN2	Copy number	(f) AATGCTTTTTAA-CATCCATATAAAGCT (r) CCTTAATTTAAG-GAATGTGAGCACC	FAM–TGATTTTGTCTA*AAA-CCC–MGBNFQ	0.76×	Anhuf *et al.*, 2003
BCKDHA	Copy number	(f) CAACCTACTCTT-CTCAGACGTGTA	(FAM/VIC)–CAGGAGATGCCCG-CCCAGCTC TAMRA	FAM: 0.18× VIC: 0.56×	DiMatteo *et al.*, 2008

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		(r) TCGAAGTGATCC-AGTGGGTAGTG			
c.815A>G	SNP	(f) TGCTGATGCTTT-GGGAAGTATGTTA	(A) (FAM/VIC)–CATGAGTGG-CTA*TCATAC–MGBNFQ	0.9×	
		(r) TGTCAGGAAAAG-ATGCTGAGTGATT	(G) FAM–ATGAGTGGCTG*TC-ATAC–MGBNFQ; VIC–CATGA-GTGGCTG*TCATAC–MGBNFQ	FAM: 0.9× VIC: 0.45×	
RNaseP	Copy number	Unknown	unknown	n/a	Standard product, 4403326, ABI

Table 2 5'-exonuclease genotyping assay design. Assay conditions in column 5 are specific to the multiplexed SMA assay. References: D. Anhuf, T. Eggermann, S. Rudnik-Schöneborn and K. Zerres, *Hum Mutat.*, 2003, **22**, 74-78; D. DiMatteo, S. Callahan and E. B. Kmiec, *Exp Cell Res.*, 2008, **15**, 878-886.

Target DNA

For some genetic targets, BCKDHA and SMN2, plasmid DNA was synthesized (GeneArt, Regensburg, Germany) containing the sequence spanning between the primers (see Table 2) and cloned into the GeneArt standard vector (2.5 kb). The target fragment was released from the cloning vector by restriction digestion with SfiI to avoid any DNA supercoiling that might affect the assay. For simplicity, these gene fragments are called "plasmid DNA" throughout the text. A string of different gene fragments was also synthesized (GeneArt) and cloned into the GeneArt standard vector for demonstration of multiplexed reactions, called an "artificial chromosome" in the text. In this case, the fragments were separated from each other by restriction digestion at flanking EcoRV sites. Human DNA was obtained in already purified form from cell lines (See Table 3; Coriell, Camden, NJ) and fragmented before use with a K7025-05 nebulizer following manufacturer's instructions (Invitrogen, Carlsbad, CA). DNA concentration was quantified by measuring absorbance at 260 nm on a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE).

Patient number	***Coriell cell line***	***Patient number, cont'd.***	***Coriell cell line, cont'd.***
1	NA14638	11	NA13714
2	NA14637	12	NA13712
3	NA14097	13	NA13709
4	NA14096	14	NA13707
5	NA14094	15	NA13705
6	NA14093	SMA carrier	NA03814
7	NA14092	SMA 1	NA03813
8	NA14091	SMA 2	NA00232
9	NA14090	SMA 3	NA09677

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10	NA13715	SMA 4	NA10684

Table 3 Map of patient numbers used in the text to Coriell cell lines.

Microfluidics

Microfluidic chips were manufactured by conventional soft lithography. Molding masters were fabricated by spin coating SU-8 negative photoresist (MicroChem Corp., Newton, MA) onto 6 inch silicon wafers and transferring the fluidic features from photomasks (CAD/Art Services, Bandon, OR) by contact lithography with an OAI Hybralign Series 200 aligner (OAI, San Jose, CA). Chips contained channels with two depths: deep channels with low hydrodynamic resistance (100 ± 10 um) for transporting fluid from external ports to the functional regions of the chip, and shallow channels (20 ± 1 um) for droplet manipulation and detection. SU-8 photoresists 2100 and 2025 were used for deep and shallow channels respectively. Polydimethylsiloxane (PDMS) (Sylgard® 184, Dow Corning, Midland, MI) chips were molded from the negative masters within mold housings of custom design. Glass cover slides were permanently bonded to the fluidic side of the chips by surface activation in an AutoGlow™ oxygen plasma system (Glow Research, Phoenix, AZ) followed by immediate contact bonding. To create hydrophobic surfaces, the microfluidic channels were exposed for ~2 min to 1H,1H,2H,2H-perfluorodecyltrichlorosilane (Alfa Aesar, Ward Hill, MA) dissolved in FC-3283 (3M Specialty Materials, St. Paul, MN) prepared as a mixture of 18 g silane in 100 uL solvent.

Two different microfluidic devices were used, one for droplet generation and the other for fluorescence readout after thermal cycling. The droplet generation chip created an emulsion of uniformly sized aqueous droplets of template DNA and PCR master mix that were suspended in an inert fluorinated oil with an emulsion stabilizing surfactant, called "carrier oil" from this point forward (REB carrier oil; RainDance Technologies, Lexington, MA). Droplets were generated in a cross-shaped microfluidic intersection, or "nozzle". As shown in Fig 3a, under typical operation the aqueous phase flowed into the nozzle from the right (160 uL/hr), joining flows of the carrier oil from the top and bottom (750 uL/hr of total oil), and producing 4 pL droplets at a rate of 11 kHz. The channel widths at the intersection measured 15 um for the aqueous inlet, 12.5 for the oil inlets, and 15 um widening to 40 um at the outlet. Flow was driven by custom OEM pumps (IDEX Corporation, Northbrook, IL).

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Approximately 25 uL of the PCR reaction mixture was collected as an emulsion from the droplet generation chip and thermally cycled in a DNA Engine (Bio-Rad, Hercules, CA). The reaction mixture contained 1× TaqMan® universal PCR master mix (Applied Biosystems, Carlsbad, CA), 0.2 mM dNTP (Takara Bio, Madison, WI), and various amounts of primer pairs and probes as described in the results. 1× assay concentration is defined as 0.2 μM probes with 0.9 μM primers. In all cases, when varied from the 1× concentration, the primers and probes were varied by the same amount. The cycler program included a 10 min hot start at 95 ℃, and 45 cycles of 15 s at 95 ℃ and 60 s at 60 ℃.

The droplets became concentrated during off-chip handling because the carrier oil is more dense than the aqueous phase and drained down from the emulsion. Hence the droplets were reinjected into the readout chip as a tightly packed emulsion that required dilution prior to readout to properly distinguish one droplet from another. A "spacer" nozzle similar to the droplet generation nozzle above was used to inject uniform plugs of extra carrier oil between droplets immediately before readout. As shown in Fig. 3b, the droplet entrance into the nozzle tapered down into a constriction about the size of an individual droplet forcing the droplets to enter the nozzle in single file and consequently at a stable rate. Opposed flow of the carrier oil from the top and bottom channels separated the droplets uniformly. The channel leaving the spacer nozzle increased in width along the direction of flow, and the droplets were interrogated by laser induced fluorescence at the location along the channel where the width was smaller than or equal to the droplet diameter (marked with an arrow in Fig. 3b). The nozzle dimensions were 15 um for the droplet entrance and exit, and 20 um for the oil lines.

Instrumentation

Fluorescence readout was performed by conventional epifluorescence microscopy with a custom microscope. A 20 mW, 488 nm laser source (Cyan; Picarro, Sunnyvale, CA) was expanded 2x and focused by the objective lens (20x / 0.45 NA; Nikon, Japan) onto the microfluidic channel. Two band pass filters discriminated the fluorescence collected through the objective lens: 512/25 nm and 529/28 nm for FAM and VIC fluorophores respectively (Semrock, Rochester, NY). Fluorescence was detected by two H5784-20 photomultipliers (Hamamatsu, Japan) and was typically recorded at a 200 kHz sampling rate with a USB-6259 data acquisition card (National Instruments, Austin, TX). The data traces were smoothed by a seven-point, second-order Savitzky-Golay algorithm before subsequent analysis. Concurrent

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with the fluorescence read out, the droplets were imaged through the same objective lens with backside illumination from an 850 nm LED (TSHG6200; Vishay Semiconductors, Shelton, CT), a short pass filter to separate the optical paths for fluorescence detection and imaging, and a Guppy CCD camera (Allied Vision Technologies, Newburyport, MA). Droplets were imaged with short illumination pulses (5-20 us) to avoid image streaking.

Data Analysis

Data was analyzed with custom LabView software (National Instruments, Austin, TX) that interpreted droplet events as contiguous bursts of fluorescence intensity above a threshold value. The signal-to-noise ratio was generally quite high and the signal levels were consistent from day to day, hence a fixed threshold value of 50 mV was used predominantly, otherwise the threshold was set by eye. The peak fluorescence intensity was recorded for each droplet event for both VIC and FAM fluorophores. Some coalescence of droplets did occur during thermal cycling, typically as isolated events between two intact droplets forming "doublets." Doublets and the rare larger coalesced events were easily filtered from the data set on based on the duration of the fluorescence burst.

Incorporation by Reference

References and citations to other documents, such as patents, patent applications, patent publications, journals, books, papers, web contents, have been made throughout this disclosure. All such documents are hereby incorporated herein by reference in their entirety for all purposes.

Equivalents

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting on the invention described herein.

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What is claimed is:

1. A microdroplet comprising a single nucleic acid template and a plurality of primer pairs specific for multiple target sites on the template.

2. The microdroplet of claim 1, further comprising a plurality of probes that hybridize to amplicons produced in said droplet.

3. The microdroplet of claim 1, wherein the single nucleic acid template is a DNA or an RNA.

4. The microdroplet of claim 1, further comprising reagents for conducting a polymerase chain reaction.

5. The microdroplet of claim 2, wherein members of the plurality of probes contain a detectable label.

6. The microdroplet of claim 6, wherein the plurality of probes comprise one or more groups of probes at varying concentrations.

7. The microdroplet of claim 6, wherein members of the one or more groups of probes each comprise the same detectable label.

8. The microdroplet of claim 5 wherein members of the plurality of probes each comprise a different detectable label.

9. The microdroplet of claim 5, wherein the detectable label is a fluorescent label.

10. A method for detecting a plurality of targets in a biological sample, said method comprising the steps of:

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a) forming one or more microdroplets each comprising a single nucleic acid template and a heterogeneous mixture of primer pairs and probes each specific for multiple target sites on the template;

b) amplifying the nucleic acid template in the one or more microdroplets; and

c) analyzing the one or more microdroplets.

11. The method of claim 10, wherein said forming steps comprises:

a) providing a first fluid comprising a single nucleic acid template;

b) providing a second fluid comprising a plurality of primers and a plurality of probes each specific for multiple target sites on the template;

c) merging the first and second fluids to form a droplet comprising the single nucleic acid template and a heterogeneous mixture of primer pairs and probes.

12. The method of claim 11, wherein the first and second fluids are merged in the presence of an electric field to said fluids.

13. The method of claim 11, wherein said first and second fluids are microdroplets.

14. The method of claim 11, wherein said second fluid further comprises reagents for conducting a polymerase chain reaction.

15. The method of claim 10, wherein the single nucleic acid template is a DNA or an RNA.

16. The method of claim 10, wherein said analyzing step comprises detecting the presence or absence of the plurality of targets in the one or more microdroplets.

17. The method of claim 10, wherein members of the plurality of probes contain a detectable label.

18. The microdroplet of claim 17, wherein the plurality of probes comprise one or more groups of probes at varying concentrations.

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19. The microdroplet of claim 18, wherein members of the one or more groups of probes each comprise the same detectable label.

20. The microdroplet of claim 17 wherein members of the plurality of probes each comprise a different detectable label.

21. The microdroplet of claim 17, wherein the detectable label is a fluorescent label.

22. A method for analyzing a target nucleic acid, the method comprising the steps of:

forming droplets containing a single target nucleic acid and one or more amplification reagents;

amplifying the target in the droplets;

excluding droplets containing amplicon from the target and amplicon from a variant of the target; and

analyzing target amplicons.

23. The method according to claim 22, wherein said amplifying step is a polymerase chain reaction and the one or more amplification reagents includes one or more primer pairs.

24. The method according to claim 22, wherein said excluding step comprises flowing said droplets in a microfluidic channel.

25. The method according to claim 22, wherein said analyzing step comprises detecting said amplicons by hybridization to detectably-labeled probes.

26. The method according to claim 22, wherein said analyzing step is conducted on amplicon from droplets that were not excluded in said excluding step.

27. The method according to claim 22, wherein said forming step comprises:

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flowing a stream of first sample fluid comprising nucleic acids such that it intersects two opposing streams of flowing carrier fluid, wherein said carrier fluid is immiscible with said sample fluid, thereby forming a plurality of first droplets comprising the first sample fluid; and

merging each of the plurality of first droplets comprising the first sample fluid with a portion of a second fluid comprising one or more amplification reagents, wherein the portion of a second fluid optionally is a droplet.

28. The method according to claim 27, wherein said carrier fluid is oil.

29. The method according to claim 28, wherein said oil comprises a surfactant.

30. The method according to claim 29, wherein the surfactant is a fluorosurfactant.

31. The method according to claim 22, wherein said analyzing step comprises:

determining a number of droplets that contain only wild-type target;

determining a number of droplets that contain only a variant of the target.

32. The method according to claim 31, wherein presence of droplets containing only said variant is indicative of a disease.

33. The method according to claim 32, wherein the disease is cancer.

34. The method according to claim 31, wherein the variant is an allelic variant.

35. The method according to claim 34, wherein the allelic variant is a single nucleotide polymorphism.

36. A method for processing nucleic acid samples, the method comprising the steps of:

obtaining droplets comprising one nucleic acid target;

amplifying said nucleic acid in said droplets; and

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separating droplets that comprise a heterogeneous population of amplicons from droplets containing a homogeneous population of amplicons.

37. A method for detecting a recurrence of a cancer in a patient, the method comprising:

forming sample droplets wherein, on average, each droplet comprises a single target nucleic acid derived from a patient sample;

flowing the sample droplets through a channel;

amplifying the target in the droplets;

detecting amplified target in the droplets;

excluding droplets comprising a heterogeneous population of amplicons; and

analyzing non-excluded droplets to determine the presence of mutant alleles indicative of recurrence.

38. The method according to claim 37, wherein said forming step comprises:

flowing a stream of sample fluid comprising nucleic acids such that it perpendicularly intersects two opposing streams of flowing carrier fluid, wherein the carrier fluid is immiscible with the sample fluid.

39. The method according to claim 37, wherein the sample is a human tissue or body fluid.

40. The method according to claim 39, wherein the body fluid is selected from the group consisting of: pus, sputum, semen, urine, blood, saliva, and cerebrospinal fluid.

41. The method of claim 37, wherein said analyzing step comprises capturing amplicon obtained from said droplets using labeled capture probes.

42. A method for analyzing a target nucleic acid, the method comprising the steps of:

compartmentalizing a first fluid into portions, each portion containing a single target nucleic acid;

amplifying the target in the portions;

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excluding portions containing amplicon from the target and amplicon from a variant of the target; and

analyzing target amplicons.

43. The method of claim 42, wherein the compartments comprise chambers in a microfluidic device.

44. A method for analyzing a target nucleic acid, the method comprising the steps of:

forming sample droplets containing a single target nucleic acid;

amplifying the target in the droplets;

excluding droplets containing amplicon from the target and amplicon from a variant of the target; and

analyzing target amplicons.

45. The method of claim 11 wherein the first fluid is contained within a first droplet and the second fluid is a first portion of a stream and further wherein step (c) comprises merging the first droplet with the first portion.

46. The method of claim 11 wherein the second fluid is contained within a first droplet and the first fluid is a first portion of a stream and further wherein step (c) comprises merging the first droplet with the first portion.

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FIGURE 1

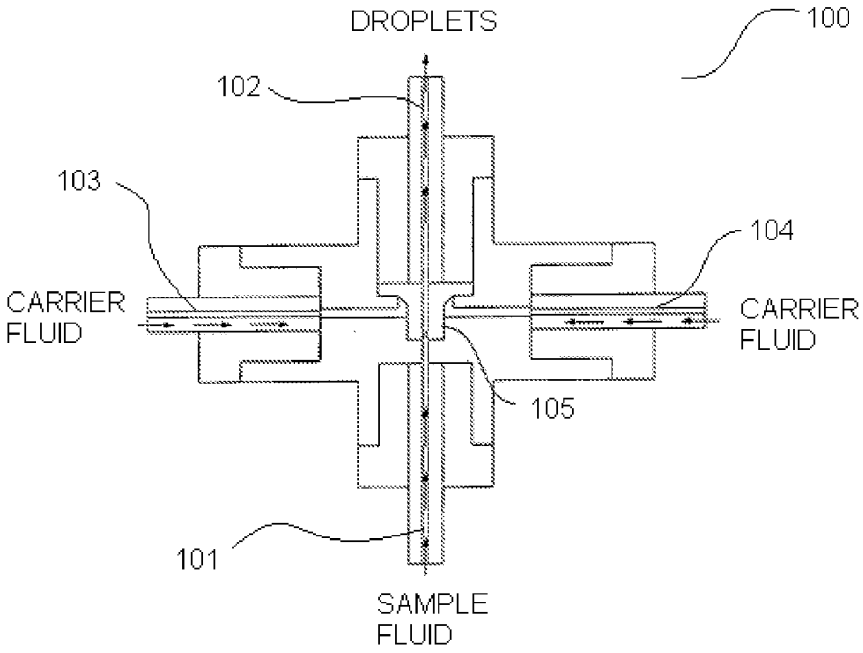
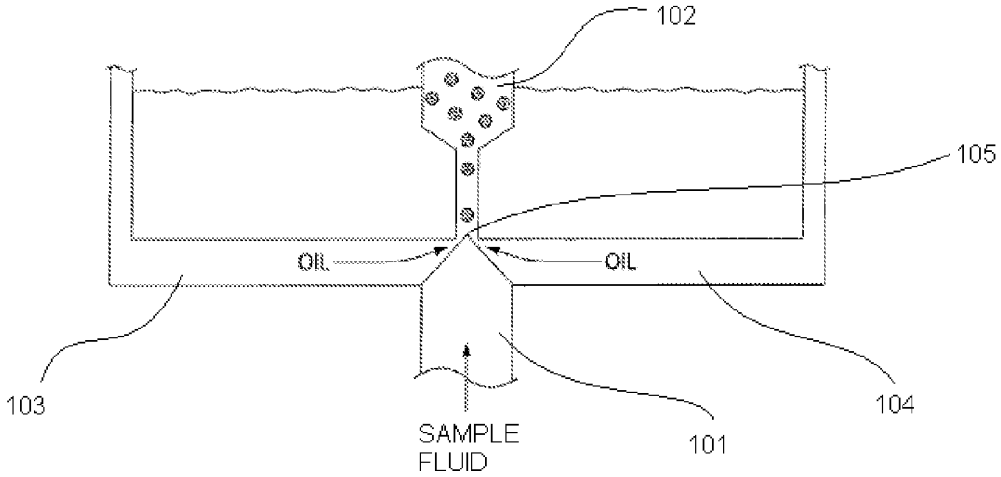
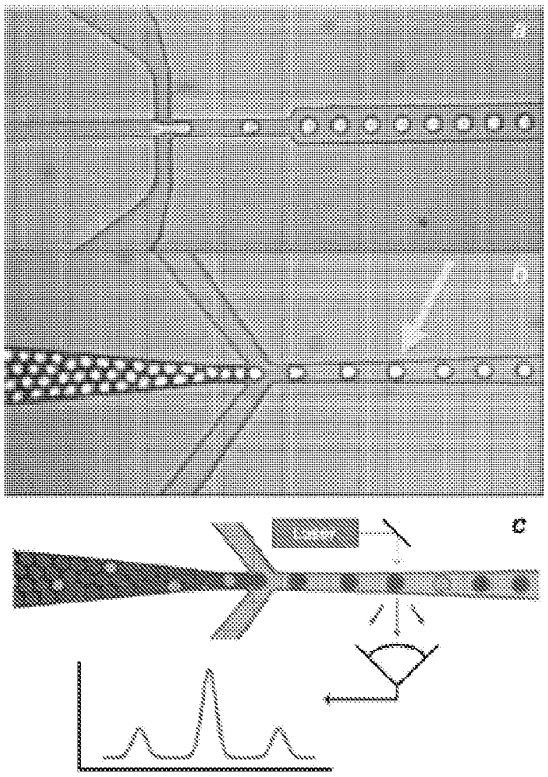


FIGURE 2



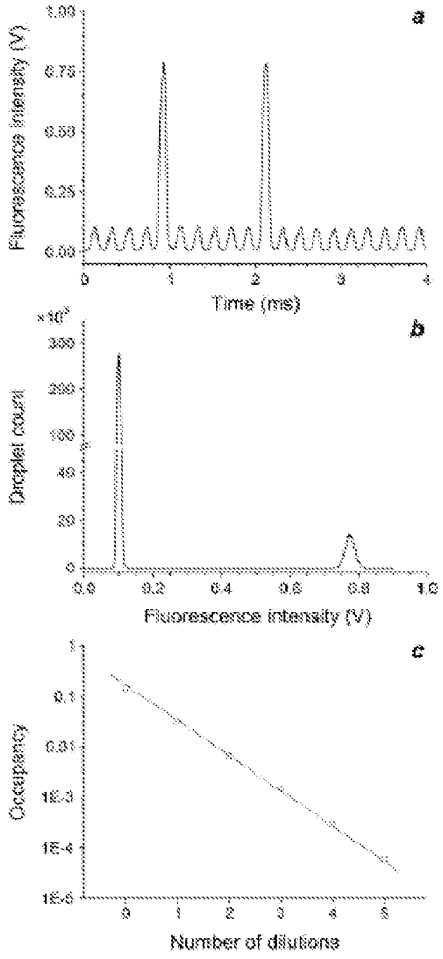
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FIGURE 3



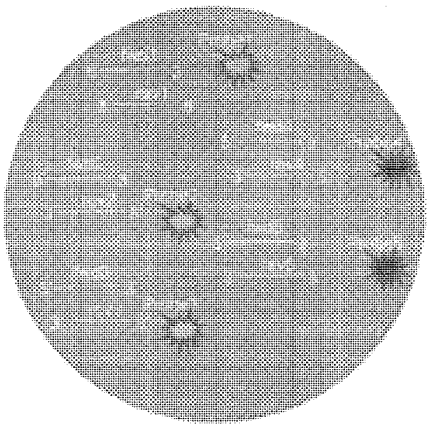
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FIGURE 4



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FIGURE 5A



- Mixture of multiple primers and probes
- 5 Taqman Assays in each droplet
  - Expression Analysis
  - Precise Quantification of copy number
  - Requires 1/6 the DNA of 5 optl-plex reactions
- Each reaction has a unique location in 2D scatter plot

FIGURE 5B

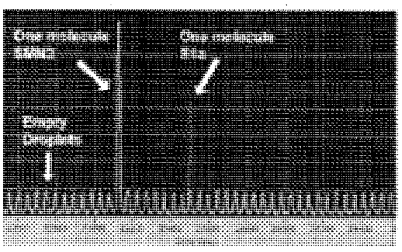
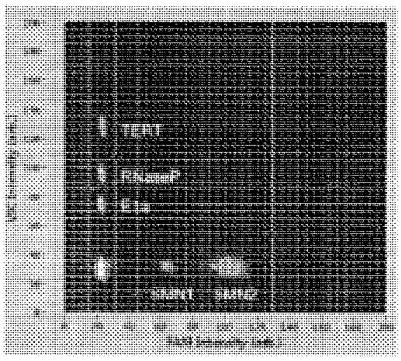
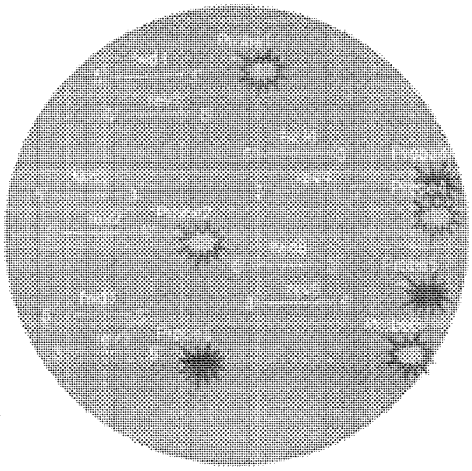


FIGURE 5C



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FIGURE 6A



- Off-axis populations are generated by making blends of probes
- Expands optical space available for multiplexing assays

FIGURE 6B

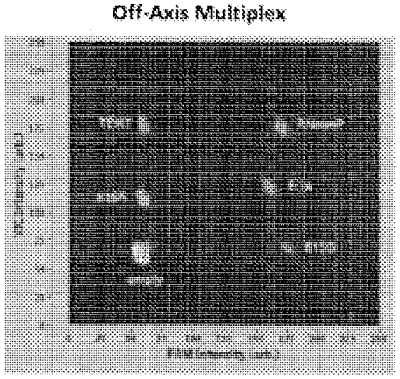
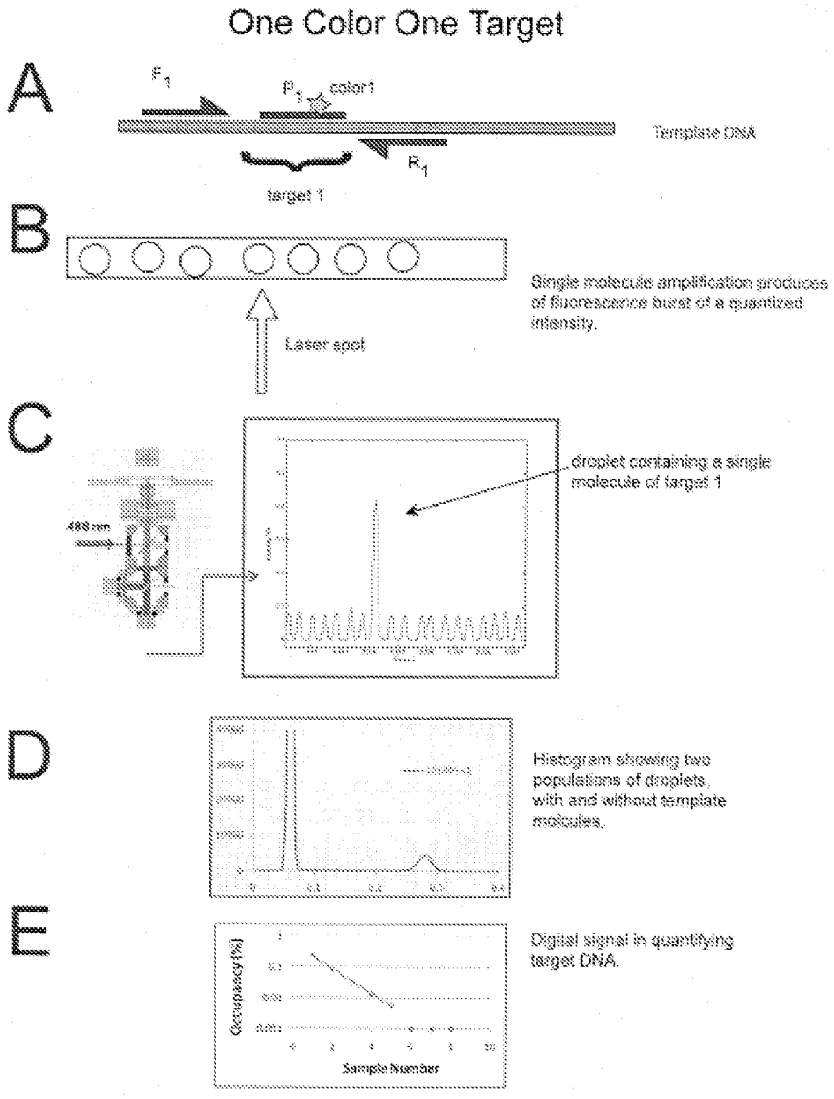


FIGURE 6C

RNaseP	2	1.01
Efa	2	0.997
815A	6	3.02
815G	1	0.497
TERT	2	1

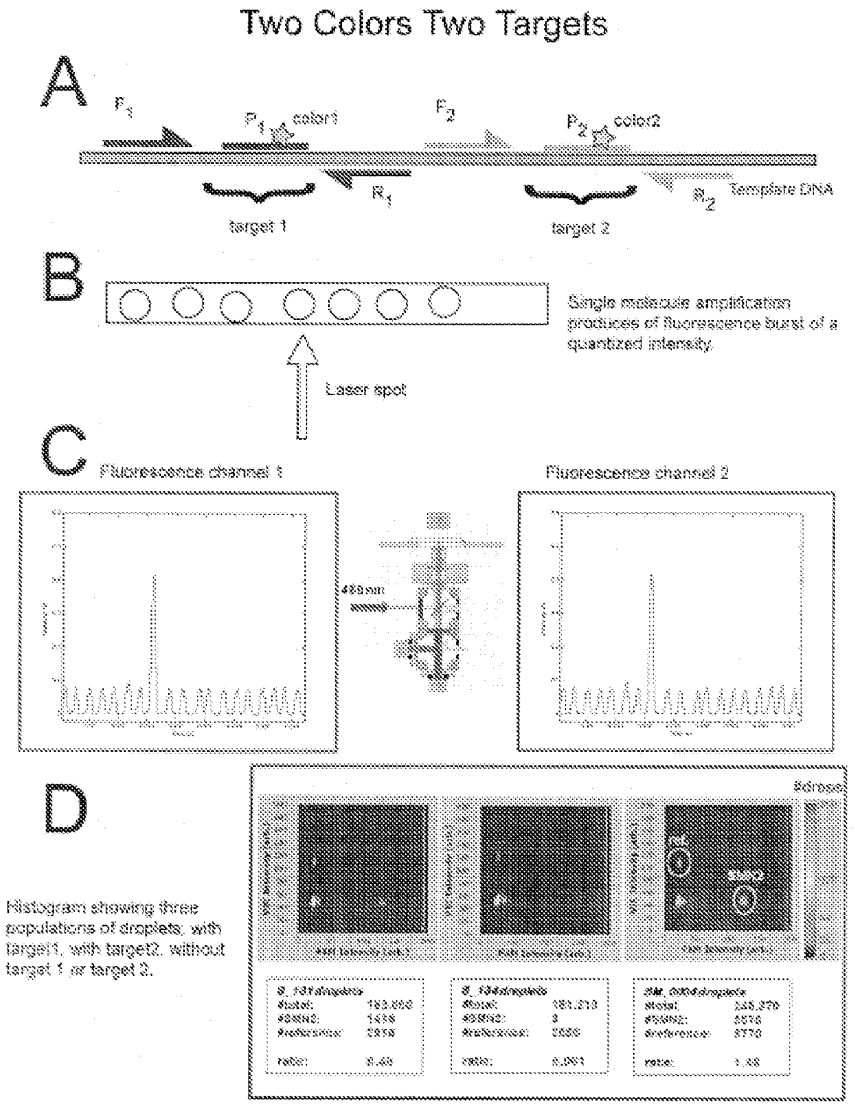
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FIGURE 7



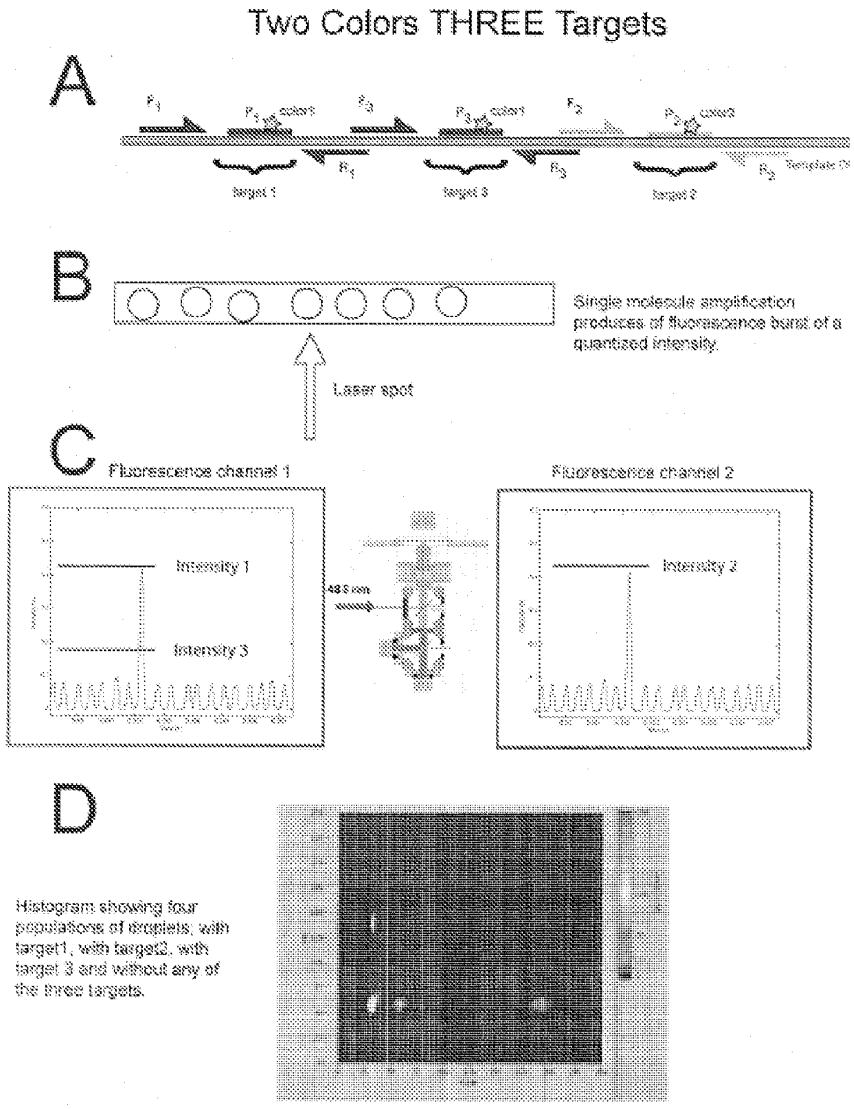
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FIGURE 8



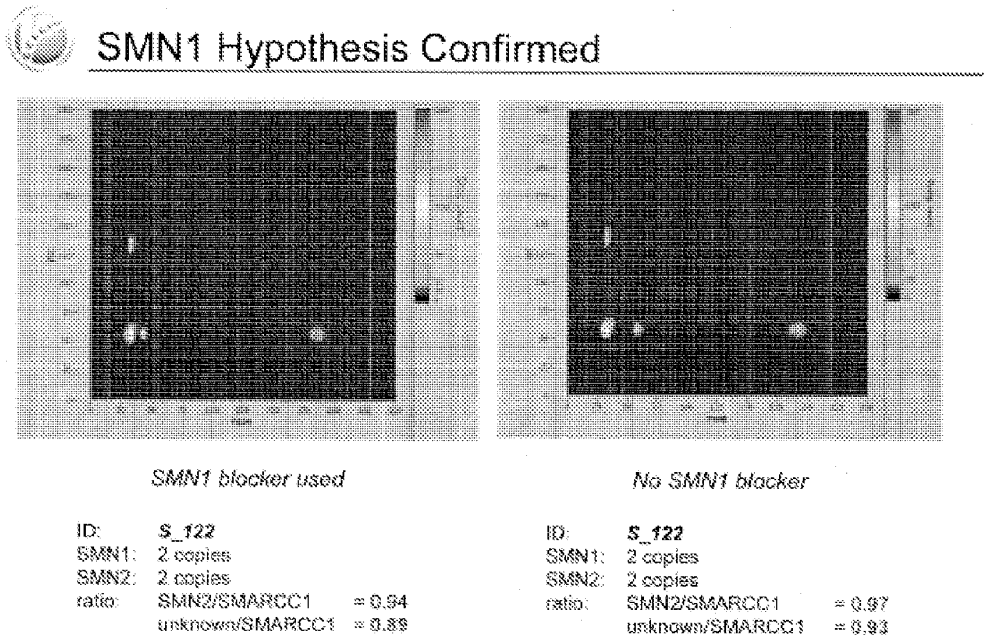
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FIGURE 9



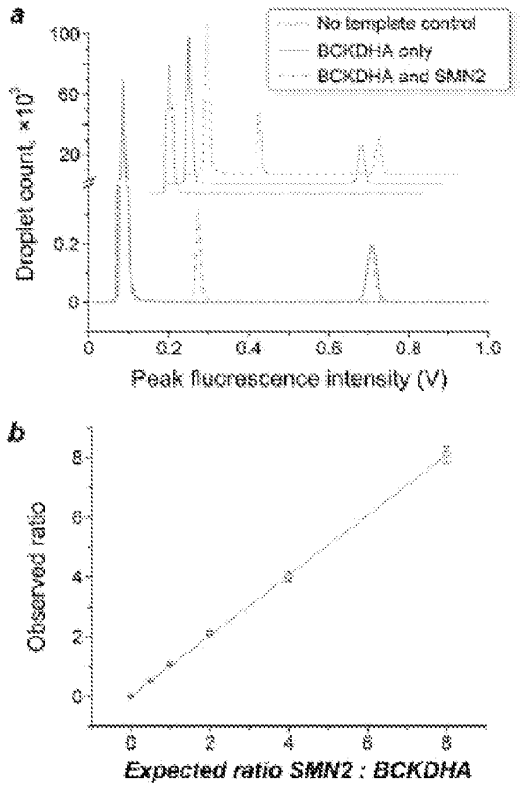
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FIGURE 10



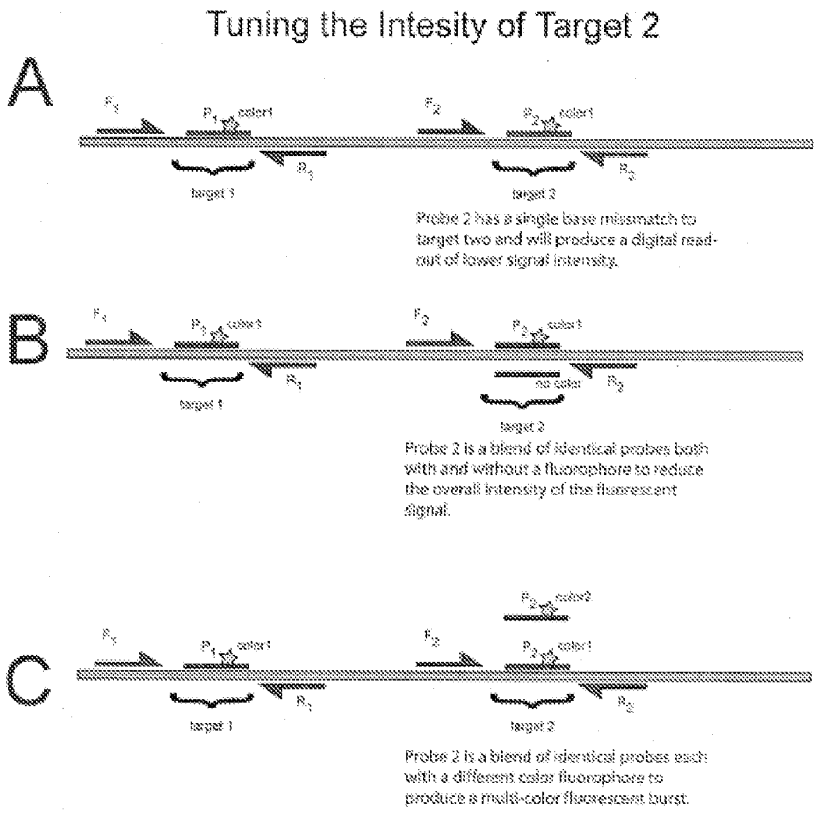
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FIGURE 11



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FIGURE 12



Relative intensity of signals from multiple targets of the same color can be generated in a variety of ways. Examples include A) using a single base missmatch of the probe and target to distinguish different targets. B) blend identical probes with and without fluorophores. C) blend identical probes with two or more different color fluorophores.

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FIGURE 13

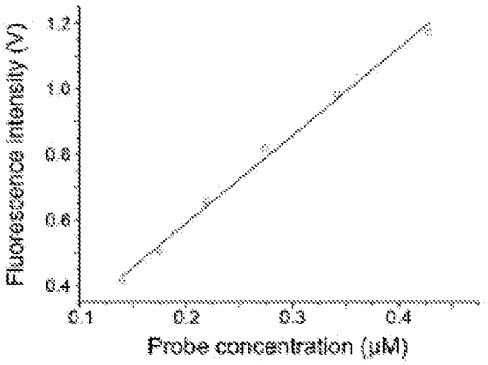
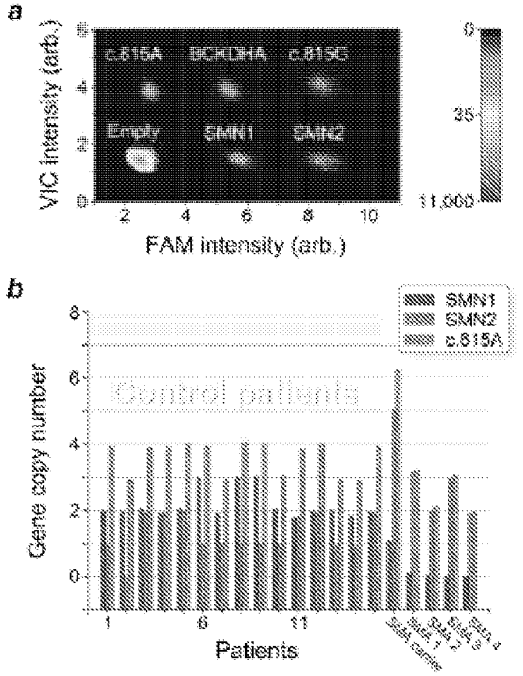


FIGURE 14



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FIGURE 15

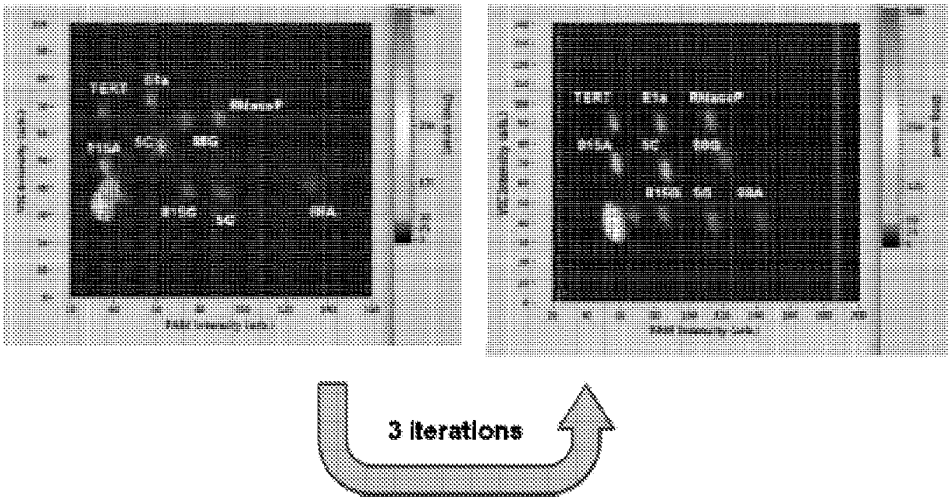
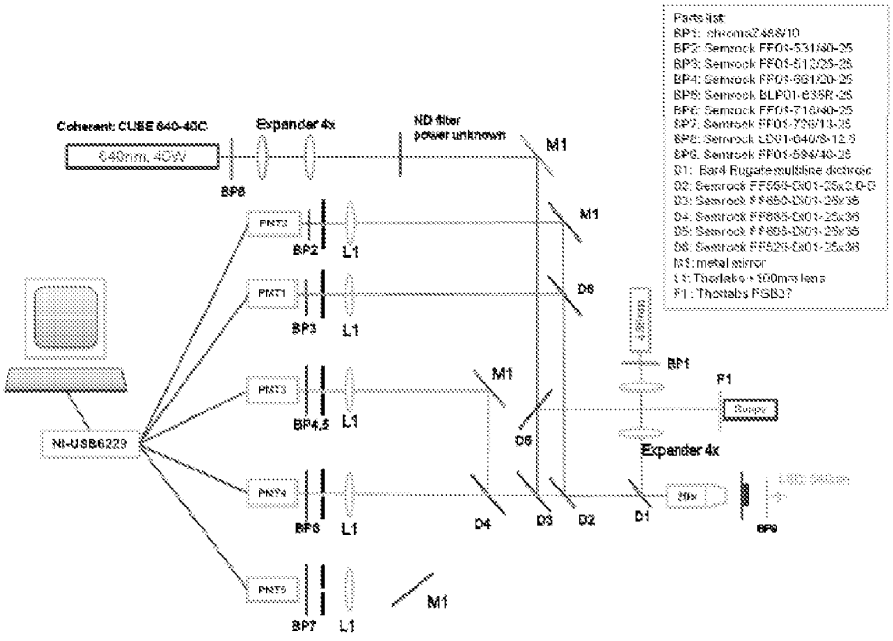
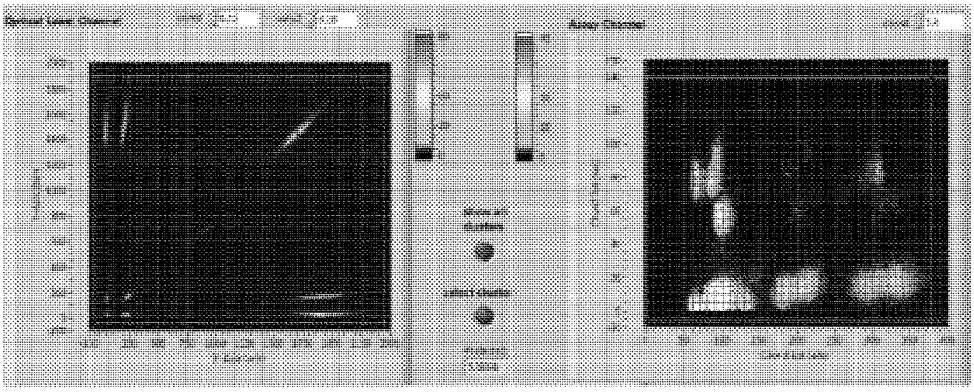


FIGURE 16



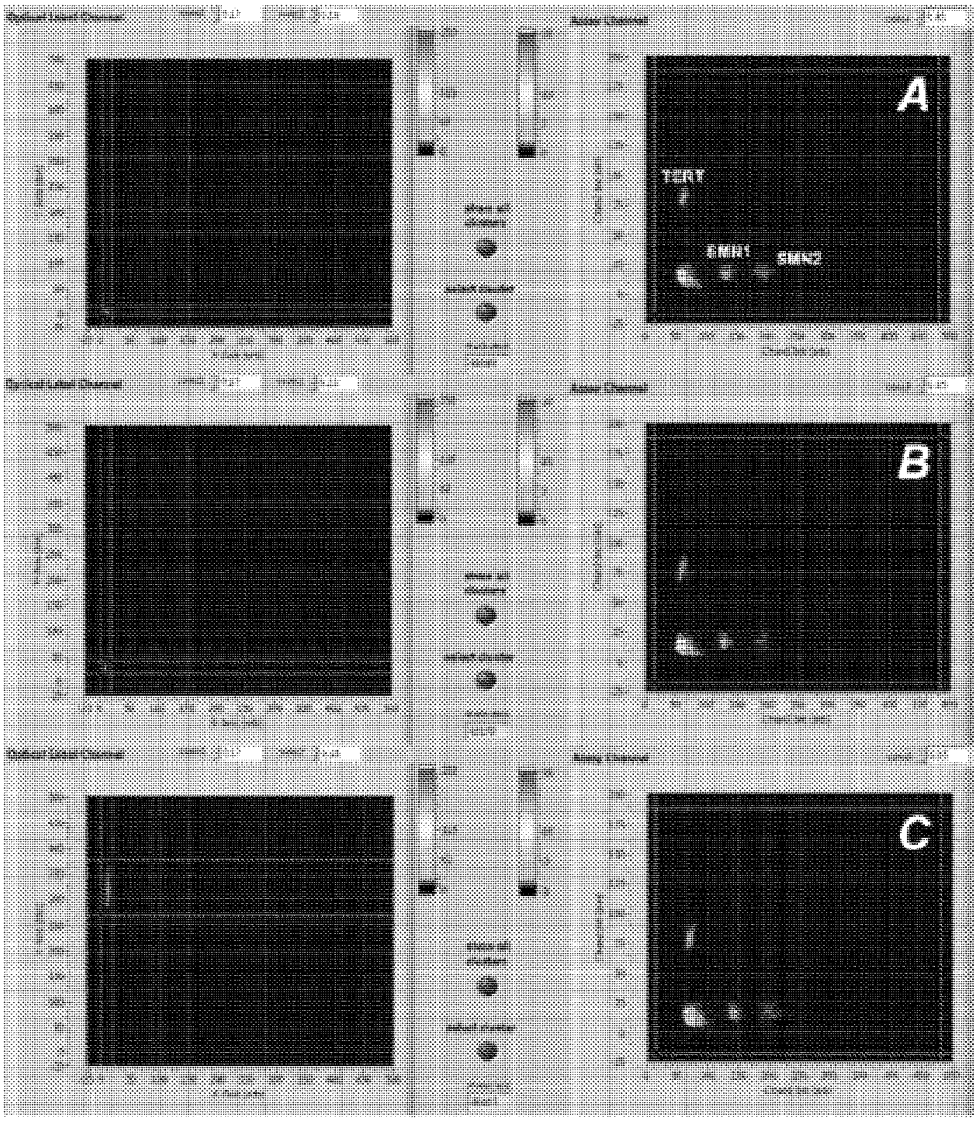
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FIGURE 17



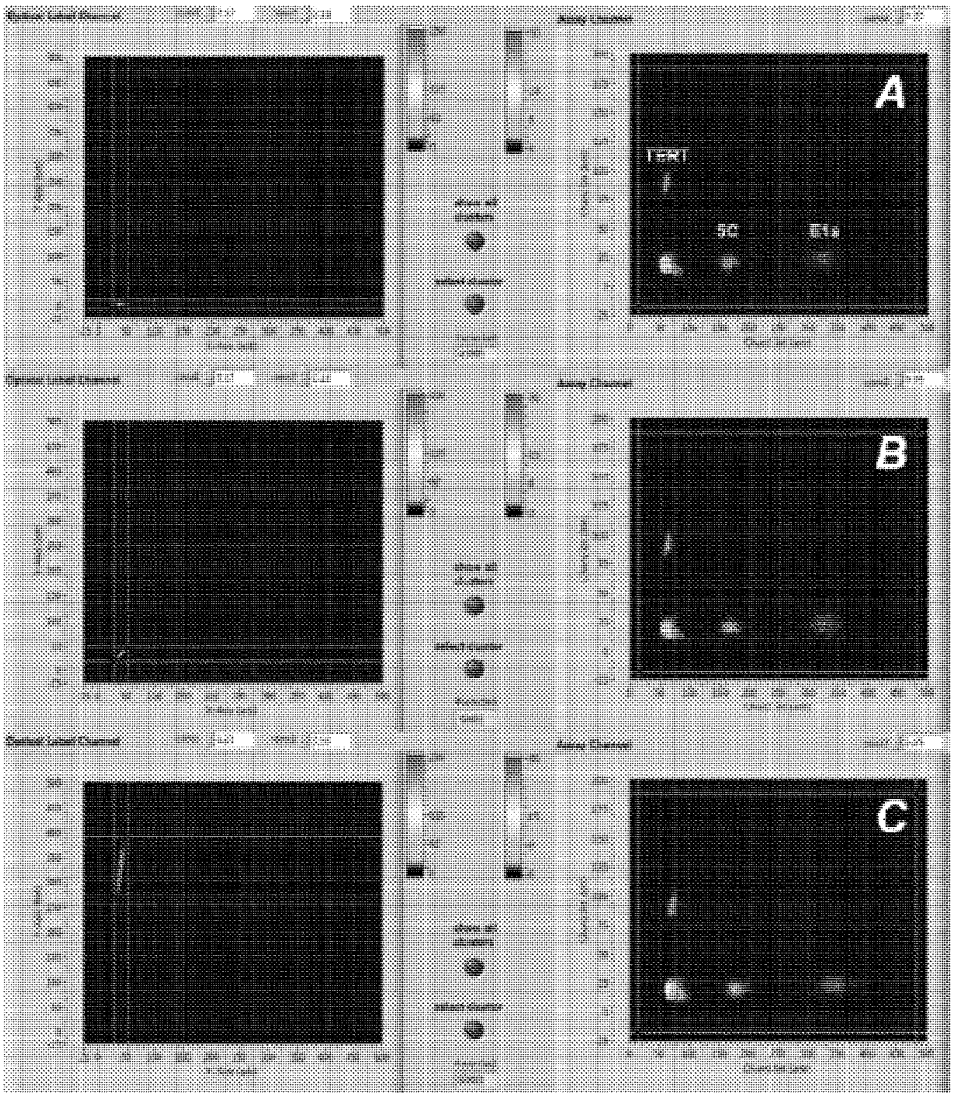
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FIGURE 18



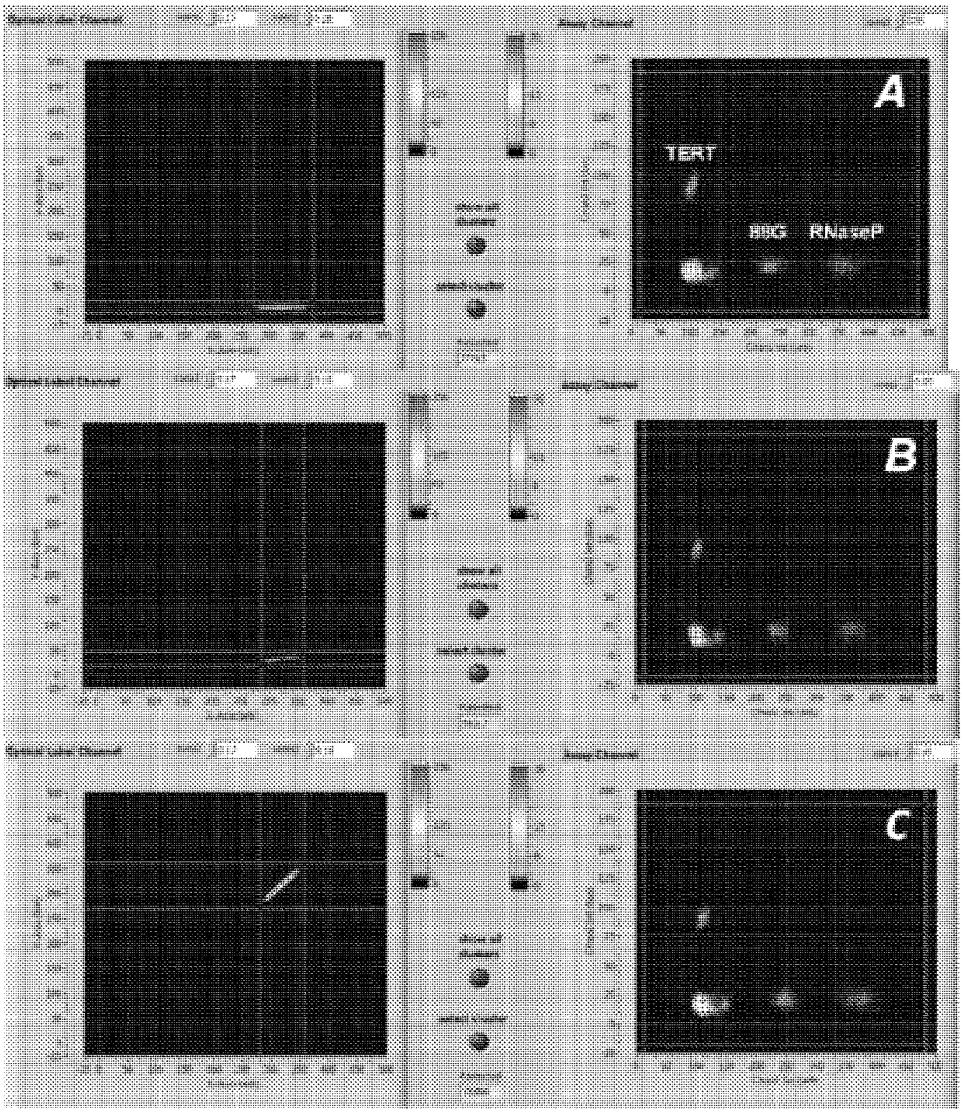
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FIGURE 19



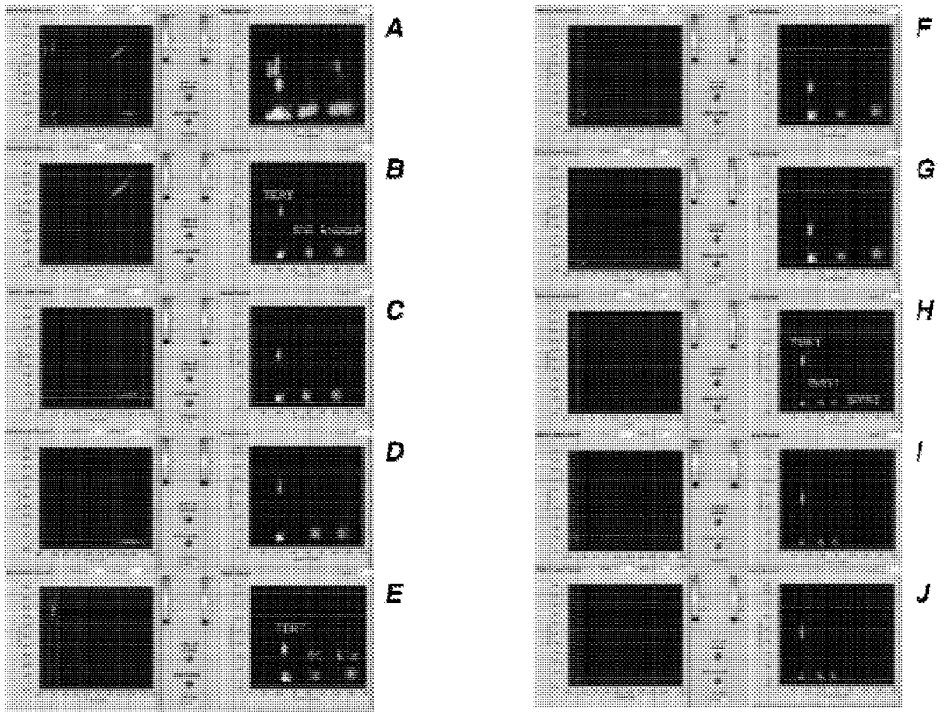
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FIGURE 20



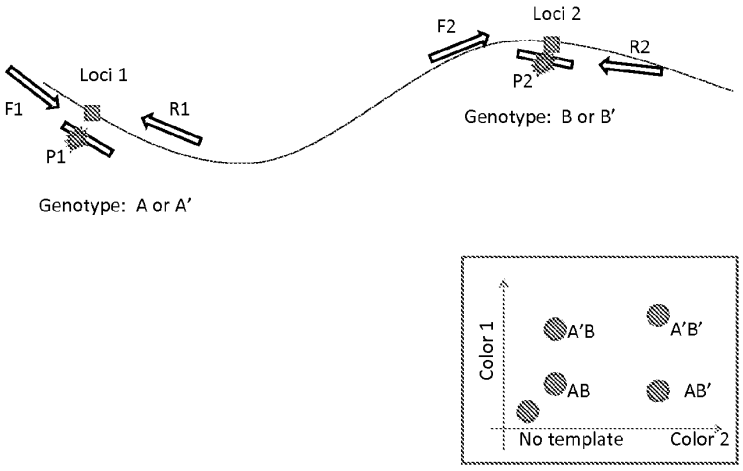
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FIGURE 21



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FIGURE 22


Loci 1
F1
R1
P1
Genotype: A or A'
F2
Loci 2
R2
P2
Genotype: B or B'
Color 1
A'B
A'B'
AB
AB'
No template
Color 2

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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/352,112	**07/24/2024 05:00:39 PM Z ET**	**88DG-373535-US**

Title of Invention

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	3145	FILED BY	Annie Wong
PATENT CENTER #	66507118	FILING DATE	07/13/2023
CUSTOMER #	30764	FIRST NAMED INVENTOR	Emil P. KARTALOV
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	David Heisey

Documents

TOTAL DOCUMENTS: 6

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
IDS-4_18352112.pdf	4	Information Disclosure Statement (IDS) Form (SB08)	598 KB
EESR_EP241618412_20Jun 2024.pdf	25	Other reference-Patent/Application/Search Documents	2978 KB
EESR_EP241618206_18Jun 2024.pdf	13	Other reference-Patent/Application/Search Documents	1418 KB
EESR_EP241618446_17Jun 2024.pdf	15	Other reference-Patent/Application/Search Documents	1692 KB

EESR_EP241618172_12Jun 2024.pdf	20	Other reference-Patent/Application/Search Documents	2351 KB
WO2011-100604A2.pdf	91	Foreign Reference	5718 KB

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If a new application is being filed and the application includes the necessary components for 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.

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NOTICE OF ALLOWANCE AND FEE(S) DUE

30764 7590 07/08/2024

Sheppard, Mullin, Richter & Hampton LLP
650 Town Center Drive, 10th Floor
Costa Mesa, CA 92626

EXAMINER	
NEGIN, RUSSELL SCOTT	
ART UNIT	PAPER NUMBER
1672	

DATE MAILED: 07/08/2024

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/352,112	07/13/2023	Emil P. KARTALOV	88DG-373535-US	3145

TITLE OF INVENTION: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	$480	$0.00	$0.00	$480	10/08/2024

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 40% the amount of undiscounted fees, and micro entity fees are 20% the amount of undiscounted fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

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PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via the USPTO patent electronic filing system.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications. **Because electronic patent issuance may occur shortly after issue fee payment, any desired continuing application should preferably be filed prior to payment of this issue fee in order not to jeopardize copendency.**

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

30764 7590 07/08/2024
Sheppard, Mullin, Richter & Hampton LLP
650 Town Center Drive, 10th Floor
Costa Mesa, CA 92626

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via the USPTO patent electronic filing system or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/352,112	07/13/2023	Emil P. KARTALOV	88DG-373535-US	3145

TITLE OF INVENTION: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	$480	$0.00	$0.00	$480	10/08/2024

EXAMINER	ART UNIT	CLASS-SUBCLASS
NEGIN, RUSSELL SCOTT	1672	702-019000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/AIA/122 or PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/AIA/47 or PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1_____
2_____
3_____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑Issue Fee ❑Publication Fee (if required)

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

❑ Electronic Payment via the USPTO patent electronic filing system ❑ Enclosed check ❑ Non-electronic payment by credit card (Attach form PTO-2038)

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

❑ Applicant asserting small entity status. See 37 CFR 1.27

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

❑ Applicant changing to regular undiscounted fee status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

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UNITED STATES PATENT AND TRADEMARK OFFICE

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

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/352,112	07/13/2023	Emil P. KARTALOV	88DG-373535-US	3145

30764 7590 07/08/2024

Sheppard, Mullin, Richter & Hampton LLP
650 Town Center Drive, 10th Floor
Costa Mesa, CA 92626

EXAMINER	
NEGIN, RUSSELL SCOTT	
ART UNIT	**PAPER NUMBER**
1672	

DATE MAILED: 07/08/2024

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

PTOL-85 (Rev. 11/23)

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OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. The United States Patent and Trademark Office (USPTO) collects the information in this record under authority of 35 U.S.C. 2. The USPTO's system of records is used to manage all applicant and owner information including name, citizenship, residence, post office address, and other information with respect to inventors and their legal representatives pertaining to the applicant's/ owner's activities in connection with the invention for which a patent is sought or has been granted. The applicable Privacy Act System of Records Notice for the information collected in this form is COMMERCE/PAT-TM-7 Patent Application Files, available in the Federal Register at 78 FR 19243 (March 29, 2013).
https://www.govinfo.gov/content/pkg/FR-2013-03-29/pdf/2013-07341.pdf

Routine uses of the information in this record may include disclosure to:

1) law enforcement, in the event that the system of records indicates a violation or potential violation of law;
2) a federal, state, local, or international agency, in response to its request;
3) a contractor of the USPTO having need for the information in order to perform a contract;
4) the Department of Justice for determination of whether the Freedom of Information Act (FOIA) requires disclosure of the record;
5) a Member of Congress submitting a request involving an individual to whom the record pertains, when the individual has requested the Member's assistance with respect to the subject matter of the record;
6) a court, magistrate, or administrative tribunal, in the course of presenting evidence, including disclosures to opposing counsel in the course of settlement negotiations;
7) the Administrator, General Services Administration (GSA), or their designee, during an inspection of records conducted by GSA under authority of 44 U.S.C. 2904 and 2906, in accordance with the GSA regulations and any other relevant (i.e., GSA or Commerce) directive, where such disclosure shall not be used to make determinations about individuals;
8) 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));
9) the Office of Personnel Management (OPM) for personnel research purposes; and
10) the Office of Management and Budget (OMB) for legislative coordination and clearance.

If you do not furnish the information requested on this form, the USPTO may not be able to process and/or examine your submission, which may result in termination of proceedings, abandonment of the application, and/or expiration of the patent.

Notice of Allowability	**Application No.** 18/352,112	**Applicant(s)** KARTALOV et al.	
	Examiner RUSSELL S NEGIN	**Art Unit** 1672	**AIA (FITF) Status** No

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

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to the communications of 5/20/2024 and 6/7/2024.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

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

3. ☑ The allowed claim(s) is/are 1-30 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

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

   Certified copies:

   a) ☐ All b) ☐ Some* c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☑ Examiner's Amendment/Comment
6. ☑ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/RUSSELL S NEGIN/
Primary Examiner, Art Unit 1672

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COMMENTS

Enter the claim amendments of 20 May 2024.

TERMINAL DISCLAIMER

The terminal disclaimer filed on 7 June 2024 disclaiming the terminal portion of any patent granted on this application which would extend beyond the expiration date of U.S. Patent 8,838,394 has been reviewed and is accepted. The terminal disclaimer has been recorded.

REASONS FOR ALLOWANCE

The following is an examiner's statement of reasons for allowance:

Even though the claims recite judicial exceptions, the combination of experimental limitations in addition to the judicial exceptions are not routine and conventional in the prior art. Consequently, the claims are subject matter eligible.

The claims are free of the prior art because the prior art does not teach experimentally decoding electromagnetic signals to determine the presence and absence patterns of analytes using the equation and values input into the equation recited in the claims.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

CONCLUSION

‘ Any inquiry concerning this communication or earlier communications from the Examiner should be directed to Russell Negin, whose telephone number is (571) 272-1083. This Examiner can normally be reached from Monday through Thursday from 8 am to 3 pm and variable hours on Fridays.

If attempts to reach the Examiner by telephone are unsuccessful, the Examiner's Supervisor, Larry Riggs, Supervisory Patent Examiner, can be reached at (571) 270-3062.

/RUSSELL S NEGIN/
Primary Examiner, Art Unit 1672
19 June 2024

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/352,112	KARTALOV et al.
	Examiner	Art Unit
	RUSSELL S NEGIN	1672

CPC - Searched*		
Symbol	Date	Examiner
C12Q1/6825 or C12Q1/68 or C12Q1/6851 or G01N21/6486 or G16B5/00 or G16B25/00 or G16B25/20 or G16B40/10 or G06F17/10 or G06F17/11 or G16B40/00	06/19/2024	RSN

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

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

Search Notes		
Search Notes	Date	Examiner
see attached search history printouts	06/19/2024	RSN
PE2E Search prior art search	06/19/2024	RSN
PE2E Search inventor search on all inventors	06/19/2024	RSN
PDP inventor search	06/19/2024	RSN
PE2E Search CPC code search with modified terms	06/19/2024	RSN

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
	see attached interference search history printouts	06/19/2024	RSN

/RUSSELL S NEGIN/ Primary Examiner, Art Unit 1672	

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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/352,112	KARTALOV et al.
	Examiner	Art Unit
	RUSSELL S NEGIN	1672

/RUSSELL S NEGIN/ Primary Examiner, Art Unit 1672	

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Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/352,112	KARTALOV et al.
	Examiner	**Art Unit**
	RUSSELL S NEGIN	1672

CPC							
Symbol						**Type**	**Version**
C12Q	/	1	/	6825		F	2013-01-01
G16B	/	25	/	00		I	2019-02-01
G16B	/	5	/	00		I	2019-02-01
C12Q	/	1	/	6851		I	2013-01-01
G16B	/	25	/	20		I	2019-02-01
G16B	/	40	/	10		I	2019-02-01
C12Q	/	1	/	68		I	2013-01-01
G01N	/	21	/	6486		I	2013-01-01
G06F	/	17	/	10		A	2013-01-01
G06F	/	17	/	11		A	2013-01-01
G16B	/	40	/	00		A	2019-02-01
Y02A	/	50	/	30		A	2018-01-01

CPC Combination Sets								
Symbol					**Type**	**Set**	**Ranking**	**Version**
C12Q	/	1	/	6851	I	1	1	2013-01-01
C12Q	/	2537	/	101	I	1	2	2013-01-01
C12Q	/	2537	/	143	I	1	3	2013-01-01
C12Q	/	2537	/	165	I	1	4	2013-01-01
C12Q	/	2563	/	107	I	1	5	2013-01-01

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	30	
/RUSSELL S NEGIN/ Primary Examiner, Art Unit 1672	19 June 2024	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	1

U.S. Patent and Trademark Office — Part of Paper No.: 20240619

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Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/352,112	KARTALOV et al.
	Examiner	**Art Unit**
	RUSSELL S NEGIN	1672

INTERNATIONAL CLASSIFICATION					
CLAIMED					
G01N	/	33	/	48	
G01N	/	33	/	50	

NON-CLAIMED					
	/		/		

US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**
702	19

CROSS REFERENCES(S)						
CLASS	**SUBCLASS (ONE SUBCLASS PER BLOCK)**					

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 30	
/RUSSELL S NEGIN/ Primary Examiner, Art Unit 1672 (Primary Examiner)	19 June 2024 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure 1

U.S. Patent and Trademark Office — Part of Paper No.: 20240619

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Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/352,112	KARTALOV et al.
	Examiner	**Art Unit**
	RUSSELL S NEGIN	1672

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

CLAIMS

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
1	1	10	9	19	19	28	28								
2	2	11	10	20	23	29	29								
3	17	12	11	21	24	30	30								
4	3	13	12	22	20										
5	4	14	13	23	21										
6	5	15	14	24	22										
7	6	16	15	25	25										
8	7	17	16	26	26										
9	8	18	18	27	27										

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	30	
/RUSSELL S NEGIN/ Primary Examiner, Art Unit 1672	19 June 2024	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	1

U.S. Patent and Trademark Office — Part of Paper No.: 20240619

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California Institute of Technology - Ex. 2026, Page 601

PE2E SEARCH - Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp
L1	3	("8838394," "10068051," "10770170," "11866768").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2023/12/30 02:19 PM
L2	0	"9376464".ap.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2023/12/30 02:26 PM
L3	16	"937464".ap.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2023/12/30 02:26 PM
L4	164	("10066263" OR "10068051" OR "10301668" OR "10770170" OR "20020022273" OR "20020146734" OR "20030134320" OR "20030148280" OR "20030148544" OR "20040053230" OR "20040191794" OR "20040248082" OR "20050053950" OR "20050064435" OR "20050106607" OR "20050164264" OR "20050214753" OR "20050250146" OR "20050260640" OR "20060039918" OR "20060216708" OR "20070072211" OR "20070178485" OR "20070231824" OR "20080050737" OR "20080069733" OR "20080096767" OR "20080124705" OR "20090042735" OR "20090048785" OR "20090062129" OR "20100015607" OR "20100041092" OR "20100047924" OR "20100112710" OR "20100120043" OR "20100151443" OR "20100159447" OR "20100210472" OR "20100233686" OR "20100248257" OR "20100261026" OR "20100267064" OR	(US-PGPUB; USPAT)	OR	ON	ON	2023/12/30 02:40 PM

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		"20100273173" OR "20100317005" OR "20100324834" OR "20110104684" OR "20110151459" OR "20110151550" OR "20110171658" OR "20110183884" OR "20110207623" OR "20110223602" OR "20110237459" OR "20120003646" OR "20120040349" OR "20120040352" OR "20120045756" OR "20120077195" OR "20120101740" OR "20120122704" OR "20120141995" OR "20120171677" OR "20120184017" OR "20120190030" OR "20120196283" OR "20120244534" OR "20120252014" OR "20120252017" OR "20120258457" OR "20120302448" OR "20130017971" OR "20130040841" OR "20130078626" OR "20130116780" OR "20130261019" OR "20130288244" OR "20140004520" OR "20140038195" OR "20140171341" OR "20140213471" OR "20140274774" OR "20150057178" OR "20150140554" OR "20150211054" OR "20150275295" OR "20160040256" OR "20160108464" OR "20160201122" OR "20160273048" OR "20170145490" OR "20170314073" OR "20170362636" OR "20180030551" OR "20180052110" OR "20180057864" OR "20190002963" OR "20190032112" OR "20190112636" OR "20190233882" OR "20200010876" OR					

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California Institute of Technology - Ex. 2026, Page 603

		"5079351" OR "5538848" OR "5618711" OR "5677152" OR "5723591" OR "5773258" OR "5789224" OR "5804375" OR "5834203" OR "5876930" OR "5882856" OR "5928862" OR "5948360" OR "5981180" OR "5994056" OR "6030787" OR "6103463" OR "6127155" OR "6171785" OR "6258569" OR "6534266" OR "6534274" OR "6548259" OR "6642062" OR "6893875" OR "7101663" OR "7141377" OR "7348141" OR "7385043" OR "7410764" OR "7413708" OR "7473767" OR "7507575" OR "7575864" OR "7667024" OR "7671184" OR "7709249" OR "7767423" OR "7771949" OR "7919237" OR "7919244" OR "7930106" OR "7941279" OR "8039215" OR "8148512" OR "8426132" OR "8455184" OR "8492094" OR "8519115" OR "8614061" OR "8771955" OR "8838394" OR "8877464" OR "8962250" OR "9133506" OR "9222128" OR "9260761" OR "9366632" OR					

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		"9422593" OR "9441266" OR "9447457" OR "9458497" OR "9791372").pn.					
L5	147	kartalov.in. NEAR emil.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)	OR	ON	ON	2023/12/30 02:42 PM
L6	299	rajagopal.in. NEAR aditya.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)	OR	ON	ON	2023/12/30 02:42 PM
L7	1102	scherer.in. NEAR axel.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)	OR	ON	ON	2023/12/30 02:42 PM
L8	245314	(C12Q1/6825 or C12Q1/68 or C12Q1/6851 or G01N21/6486 or G16B5/00 or G16B25/00 or	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB,	OR	ON	ON	2023/12/30 02:43 PM

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		G16B25/20 or G16B40/10 or G06F17/10 or G06F17/11 or G16B40/00).cpc.	HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
L9	1302	L5 L6 L7	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:43 PM
L10	124	L8 AND L9	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:43 PM
L11	669450	analytes	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT;	OR	ON	ON	2023/12/30 02:44 PM

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			IBM_TDB)				
L12	74	L10 AND L11	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:44 PM
L13	24385663	channels	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:44 PM
L14	49	L12 AND L13	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:44 PM
L15	7800095	wavelength	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS,	OR	ON	ON	2023/12/30 02:44 PM

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			IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
L16	37838	L15 AND L8	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:44 PM
L17	69	L9 AND L16	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:44 PM
L18	368464	fluorophores	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:44 PM

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L19	66	L17 AND L18	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:44 PM
L20	9645392	detector	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:45 PM
L21	53	L19 AND L20	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:45 PM
L22	1	("11009486").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2023/12/31 07:15 AM
L23	1	("20110264420").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2023/12/31 02:49 PM
L24	4	("8838394," "10068051," "10770170," "11866768").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/02/03 11:31 AM

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L25	0	"9376464".ap.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/02/03 11:31 AM
L26	17	"937464".ap.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/02/03 11:31 AM
L27	164	("10066263" OR "10068051" OR "10301668" OR "10770170" OR "20020022273" OR "20020146734" OR "20030134320" OR "20030148280" OR "20030148544" OR "20040053230" OR "20040191794" OR "20040248082" OR "20050053950" OR "20050064435" OR "20050106607" OR "20050164264" OR "20050214753" OR "20050250146" OR "20050260640" OR "20060039918" OR "20060216708" OR "20070072211" OR "20070178485" OR "20070231824" OR "20080050737" OR "20080069733" OR "20080096767" OR "20080124705" OR "20090042735" OR "20090048785" OR "20090062129" OR "20100015607" OR "20100041092" OR "20100047924" OR "20100112710" OR "20100120043" OR "20100151443" OR "20100159447" OR "20100210472" OR "20100233686" OR "20100248257" OR "20100261026" OR "20100267064" OR "20100273173" OR "20100317005" OR "20100324834" OR "20110104684" OR "20110151459" OR "20110151550" OR "20110171658" OR "20110183884" OR "20110207623" OR "20110223602" OR	(US-PGPUB; USPAT)	OR	ON	ON	2024/02/03 11:31 AM

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		"20110237459" OR "20120003646" OR "20120040349" OR "20120040352" OR "20120045756" OR "20120077195" OR "20120101740" OR "20120122704" OR "20120141995" OR "20120171677" OR "20120184017" OR "20120190030" OR "20120196283" OR "20120244534" OR "20120252014" OR "20120252017" OR "20120258457" OR "20120302448" OR "20130017971" OR "20130040841" OR "20130078626" OR "20130116780" OR "20130261019" OR "20130288244" OR "20140004520" OR "20140038195" OR "20140171341" OR "20140213471" OR "20140274774" OR "20150057178" OR "20150140554" OR "20150211054" OR "20150275295" OR "20160040256" OR "20160108464" OR "20160201122" OR "20160273048" OR "20170145490" OR "20170314073" OR "20170362636" OR "20180030551" OR "20180052110" OR "20180057864" OR "20190002963" OR "20190032112" OR "20190112636" OR "20190233882" OR "20200010876" OR "5079351" OR "5538848" OR "5618711" OR "5677152" OR "5723591" OR "5773258" OR "5789224" OR "5804375" OR "5834203" OR "5876930" OR					

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		"5882856" OR "5928862" OR "5948360" OR "5981180" OR "5994056" OR "6030787" OR "6103463" OR "6127155" OR "6171785" OR "6258569" OR "6534266" OR "6534274" OR "6548259" OR "6642062" OR "6893875" OR "7101663" OR "7141377" OR "7348141" OR "7385043" OR "7410764" OR "7413708" OR "7473767" OR "7507575" OR "7575864" OR "7667024" OR "7671184" OR "7709249" OR "7767423" OR "7771949" OR "7919237" OR "7919244" OR "7930106" OR "7941279" OR "8039215" OR "8148512" OR "8426132" OR "8455184" OR "8492094" OR "8519115" OR "8614061" OR "8771955" OR "8838394" OR "8877464" OR "8962250" OR "9133506" OR "9222128" OR "9260761" OR "9366632" OR "9422593" OR "9441266" OR "9447457" OR "9458497" OR "9791372").pn.					
L28	151	kartalov.in. NEAR emil.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU,	OR	ON	ON	2024/02/03 11:31 AM

			CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)				
L29	304	rajagopal.in. NEAR aditya.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)	OR	ON	ON	2024/02/03 11:31 AM
L30	1105	scherer.in. NEAR axel.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)	OR	ON	ON	2024/02/03 11:31 AM
L31	246800	(C12Q1/6825 or C12Q1/68 or C12Q1/6851 or G01N21/6486 or G16B5/00 or G16B25/00 or G16B25/20 or G16B40/10 or G06F17/10 or G06F17/11 or G16B40/00).cpc.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM

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L32	1309	L5 L6 L7	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM
L33	127	L8 AND L9	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM
L34	674085	analytes	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM
L35	77	L10 AND L11	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL,	OR	ON	ON	2024/02/03 11:31 AM

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			NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
L36	24551447	channels	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM
L37	52	L12 AND L13	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM
L38	7848544	wavelength	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM
L39	38061	L15 AND L8	(US-PGPUB; USPAT;	OR	ON	ON	2024/02/03

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			USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)				11:31 AM
L40	72	L9 AND L16	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM
L41	371430	fluorophores	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM
L42	69	L17 AND L18	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL,	OR	ON	ON	2024/02/03 11:31 AM

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			PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
L43	9696276	detector	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM
L44	56	L19 AND L20	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM
L45	1	("11009486").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/02/03 11:31 AM
L46	1	("20110264420").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/02/03 11:31 AM
L47	4	("8838394," "10068051," "10770170," "11866768").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/05/27 12:48 PM
L48	3	("20050079514," "20130045873," "20060088876").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/01 09:20 AM
L49	100	defb8 OR hamp2	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/01 09:20 AM
L50	0	L48 AND L49	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/01 09:20 AM
L51	1	("10441517").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/01 12:45 PM

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L52	4	("8838394," "10068051," "10770170," "11866768").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/19 10:55 AM
L53	0	"9376464".ap.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/19 10:55 AM
L54	18	"937464".ap.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/19 10:55 AM
L55	164	("10066263" OR "10068051" OR "10301668" OR "10770170" OR "20020022273" OR "20020146734" OR "20030134320" OR "20030148280" OR "20030148544" OR "20040053230" OR "20040191794" OR "20040248082" OR "20050053950" OR "20050064435" OR "20050106607" OR "20050164264" OR "20050214753" OR "20050250146" OR "20050260640" OR "20060039918" OR "20060216708" OR "20070072211" OR "20070178485" OR "20070231824" OR "20080050737" OR "20080069733" OR "20080096767" OR "20080124705" OR "20090042735" OR "20090048785" OR "20090062129" OR "20100015607" OR "20100041092" OR "20100047924" OR "20100112710" OR "20100120043" OR "20100151443" OR "20100159447" OR "20100210472" OR "20100233686" OR "20100248257" OR "20100261026" OR "20100267064" OR "20100273173" OR "20100317005" OR "20100324834" OR "20110104684" OR "20110151459" OR "20110151550" OR	(US-PGPUB; USPAT)	OR	ON	ON	2024/06/19 10:55 AM

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		"20110171658" OR "20110183884" OR "20110207623" OR "20110223602" OR "20110237459" OR "20120003646" OR "20120040349" OR "20120040352" OR "20120045756" OR "20120077195" OR "20120101740" OR "20120122704" OR "20120141995" OR "20120171677" OR "20120184017" OR "20120190030" OR "20120196283" OR "20120244534" OR "20120252014" OR "20120252017" OR "20120258457" OR "20120302448" OR "20130017971" OR "20130040841" OR "20130078626" OR "20130116780" OR "20130261019" OR "20130288244" OR "20140004520" OR "20140038195" OR "20140171341" OR "20140213471" OR "20140274774" OR "20150057178" OR "20150140554" OR "20150211054" OR "20150275295" OR "20160040256" OR "20160108464" OR "20160201122" OR "20160273048" OR "20170145490" OR "20170314073" OR "20170362636" OR "20180030551" OR "20180052110" OR "20180057864" OR "20190002963" OR "20190032112" OR "20190112636" OR "20190233882" OR "20200010876" OR "5079351" OR "5538848" OR "5618711" OR "5677152" OR "5723591" OR "5773258" OR					

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		"5789224" OR "5804375" OR "5834203" OR "5876930" OR "5882856" OR "5928862" OR "5948360" OR "5981180" OR "5994056" OR "6030787" OR "6103463" OR "6127155" OR "6171785" OR "6258569" OR "6534266" OR "6534274" OR "6548259" OR "6642062" OR "6893875" OR "7101663" OR "7141377" OR "7348141" OR "7385043" OR "7410764" OR "7413708" OR "7473767" OR "7507575" OR "7575864" OR "7667024" OR "7671184" OR "7709249" OR "7767423" OR "7771949" OR "7919237" OR "7919244" OR "7930106" OR "7941279" OR "8039215" OR "8148512" OR "8426132" OR "8455184" OR "8492094" OR "8519115" OR "8614061" OR "8771955" OR "8838394" OR "8877464" OR "8962250" OR "9133506" OR "9222128" OR "9260761" OR "9366632" OR "9422593" OR "9441266" OR "9447457" OR "9458497" OR "9791372").pn.					

L56	157	kartalov.in. NEAR emil.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)	OR	ON	ON	2024/06/19 10:55 AM
L57	320	rajagopal.in. NEAR aditya.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)	OR	ON	ON	2024/06/19 10:55 AM
L58	1124	scherer.in. NEAR axel.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)	OR	ON	ON	2024/06/19 10:55 AM
L59	258384	(C12Q1/6825 or C12Q1/68 or C12Q1/6851 or G01N21/6486 or G16B5/00 or G16B25/00 or G16B25/20 or G16B40/10 or G06F17/10 or G06F17/11 or G16B40/00).cpc.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK,	OR	ON	ON	2024/06/19 10:55 AM

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			TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
L60	1336	L5 L6 L7	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L61	140	L8 AND L9	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L62	693002	analytes	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L63	86	L10 AND L11	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU,	OR	ON	ON	2024/06/19 10:55 AM

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			CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
L64	25200697	channels	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L65	61	L12 AND L13	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L66	8043112	wavelength	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN,	OR	ON	ON	2024/06/19 10:55 AM

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			WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
L67	39673	L15 AND L8	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L68	85	L9 AND L16	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L69	383248	fluorophores	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L70	82	L17 AND L18	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA,	OR	ON	ON	2024/06/19 10:55 AM

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			EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
L71	9898965	detector	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L72	66	L19 AND L20	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L73	1	("11009486").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/19 10:55 AM
L74	1	("20110264420").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/19 10:55 AM
L75	4	("8838394," "10068051," "10770170," "11866768").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/19 10:55 AM
L76	0	"9376464".ap.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/19 10:55 AM
L77	18	"937464".ap.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/19 10:55 AM

L78	164	("10066263" OR "10068051" OR "10301668" OR "10770170" OR "20020022273" OR "20020146734" OR "20030134320" OR "20030148280" OR "20030148544" OR "20040053230" OR "20040191794" OR "20040248082" OR "20050053950" OR "20050064435" OR "20050106607" OR "20050164264" OR "20050214753" OR "20050250146" OR "20050260640" OR "20060039918" OR "20060216708" OR "20070072211" OR "20070178485" OR "20070231824" OR "20080050737" OR "20080069733" OR "20080096767" OR "20080124705" OR "20090042735" OR "20090048785" OR "20090062129" OR "20100015607" OR "20100041092" OR "20100047924" OR "20100112710" OR "20100120043" OR "20100151443" OR "20100159447" OR "20100210472" OR "20100233686" OR "20100248257" OR "20100261026" OR "20100267064" OR "20100273173" OR "20100317005" OR "20100324834" OR "20110104684" OR "20110151459" OR "20110151550" OR "20110171658" OR "20110183884" OR "20110207623" OR "20110223602" OR "20110237459" OR "20120003646" OR "20120040349" OR "20120040352" OR "20120045756" OR	(US-PGPUB; USPAT)	OR	ON	ON	2024/06/19 10:55 AM

		"20120077195" OR "20120101740" OR "20120122704" OR "20120141995" OR "20120171677" OR "20120184017" OR "20120190030" OR "20120196283" OR "20120244534" OR "20120252014" OR "20120252017" OR "20120258457" OR "20120302448" OR "20130017971" OR "20130040841" OR "20130078626" OR "20130116780" OR "20130261019" OR "20130288244" OR "20140004520" OR "20140038195" OR "20140171341" OR "20140213471" OR "20140274774" OR "20150057178" OR "20150140554" OR "20150211054" OR "20150275295" OR "20160040256" OR "20160108464" OR "20160201122" OR "20160273048" OR "20170145490" OR "20170314073" OR "20170362636" OR "20180030551" OR "20180052110" OR "20180057864" OR "20190002963" OR "20190032112" OR "20190112636" OR "20190233882" OR "20200010876" OR "5079351" OR "5538848" OR "5618711" OR "5677152" OR "5723591" OR "5773258" OR "5789224" OR "5804375" OR "5834203" OR "5876930" OR "5882856" OR "5928862" OR "5948360" OR "5981180" OR "5994056" OR					

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		"6030787" OR "6103463" OR "6127155" OR "6171785" OR "6258569" OR "6534266" OR "6534274" OR "6548259" OR "6642062" OR "6893875" OR "7101663" OR "7141377" OR "7348141" OR "7385043" OR "7410764" OR "7413708" OR "7473767" OR "7507575" OR "7575864" OR "7667024" OR "7671184" OR "7709249" OR "7767423" OR "7771949" OR "7919237" OR "7919244" OR "7930106" OR "7941279" OR "8039215" OR "8148512" OR "8426132" OR "8455184" OR "8492094" OR "8519115" OR "8614061" OR "8771955" OR "8838394" OR "8877464" OR "8962250" OR "9133506" OR "9222128" OR "9260761" OR "9366632" OR "9422593" OR "9441266" OR "9447457" OR "9458497" OR "9791372").pn.					
L79	157	kartalov.in. NEAR emil.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO,	OR	ON	ON	2024/06/19 10:55 AM

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			MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)				
L80	320	rajagopal.in. NEAR aditya.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)	OR	ON	ON	2024/06/19 10:55 AM
L81	1124	scherer.in. NEAR axel.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)	OR	ON	ON	2024/06/19 10:55 AM
L82	258384	(C12Q1/6825 or C12Q1/68 or C12Q1/6851 or G01N21/6486 or G16B5/00 or G16B25/00 or G16B25/20 or G16B40/10 or G06F17/10 or G06F17/11 or G16B40/00).cpc.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L83	1336	L5 L6 L7	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU,	OR	ON	ON	2024/06/19 10:55 AM

			CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
L84	140	L8 AND L9	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L85	693002	analytes	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L86	86	L10 AND L11	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN,	OR	ON	ON	2024/06/19 10:55 AM

IPR2025-01546
California Institute of Technology - Ex. 2026, Page 630

			WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
L87	25200697	channels	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L88	61	L12 AND L13	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L89	8043112	wavelength	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L90	39673	L15 AND L8	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA,	OR	ON	ON	2024/06/19 10:55 AM

IPR2025-01546
California Institute of Technology - Ex. 2026, Page 631

			EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
L91	85	L9 AND L16	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L92	383248	fluorophores	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L93	82	L17 AND L18	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO;	OR	ON	ON	2024/06/19 10:55 AM

IPR2025-01546
California Institute of Technology - Ex. 2026, Page 632

			JPO; DERWENT; IBM_TDB)				
L94	9898965	detector	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L95	66	L19 AND L20	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/06/19 10:55 AM
L96	1	("11009486").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/19 10:55 AM
L97	1	("20110264420").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/19 10:55 AM
L98	4	("8838394," "10068051," "10770170," "11866768").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/19 10:55 AM
L99	3	("20050079514," "20130045873," "20060088876").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/19 10:55 AM
L100	100	defb8 OR hamp2	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/19 10:55 AM
L101	0	L48 AND L49	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/19 10:55 AM
L102	1	("10441517").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/06/19 10:55 AM

PE2E SEARCH - Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp
N1	1638689	wavelength	(US-PGPUB; USPAT)	OR	ON	ON	2024/06/19 10:53 AM
N2	413010	cumulative	(US-PGPUB; USPAT)	OR	ON	ON	2024/06/19 10:53 AM
N3	65345	N1 AND N2	(US-PGPUB; USPAT)	OR	ON	ON	2024/06/19 10:54 AM
N4	133465	fluorophore	(US-PGPUB; USPAT)	OR	ON	ON	2024/06/19 10:54 AM
N5	7003	N3 AND N4	(US-PGPUB; USPAT)	OR	ON	ON	2024/06/19 10:54 AM
N6	4423180	channels	(US-PGPUB; USPAT)	OR	ON	ON	2024/06/19 10:54 AM
N7	4235	N5 AND N6	(US-PGPUB; USPAT)	OR	ON	ON	2024/06/19 10:54 AM
N8	190616	analytes	(US-PGPUB; USPAT)	OR	ON	ON	2024/06/19 10:55 AM
N9	1801	N7 AND N8	(US-PGPUB; USPAT)	OR	ON	ON	2024/06/19 10:55 AM
N10	773765	decoding	(US-PGPUB; USPAT)	OR	ON	ON	2024/06/19 10:55 AM
N11	306	N9 AND N10	(US-PGPUB; USPAT)	OR	ON	ON	2024/06/19 10:55 AM

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18352112
Google Scholar Search
/RSN/ 6/19/2024

probes hybridization fluorophores
probes hybridization fluorophores wavelengths
probes hybridization fluorophores wavelengths channels
probes hybridization fluorophores wavelengths channels decoding
probes hybridization fluorophores wavelengths channels decoding cumulative
probes hybridization fluorophores wavelengths channels decoding component
probes hybridization fluorophores wavelengths channels decoding analytes

IPR2025-01546
California Institute of Technology - Ex. 2026, Page 635

PATENT
Docket No.: 88DG-373535-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	)	
	)	Confirmation No.: 3145
Emil P. KARTALOV *et al.*	)	
	)	Examiner: Russell S. NEGIN
Application No.: 18/352,112	)	
	)	
Filed: July 13, 2023	)	Art Unit: 1672
	)	
For: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	)	***VIA Patent Center***

OK TO ENTER
/RSN/ 6/19/2024

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

Commissioner:

RESPONSE TO FINAL OFFICE ACTION

In response to the final Office Action mailed February 12, 2024, the period for response ending on June 12, 2024, being submitted with payment for a one-month extension of time, Applicant respectfully requests the Examiner to reconsider this Application in light of the following.

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

Remarks begin on page 10 of this paper.

Bibliographic Data

Application No: 18/352,112

Foreign Priority claimed: ○ Yes ◉ No

35 USC 119 (a-d) conditions met: ☐ Yes ☑ No ☐ Met After Allowance

Verified and Acknowledged: /Russell Negin/ (Examiner's Signature) RSN (Initials)

Title: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
07/13/2023	702	1672	88DG-373535-US
RULE			

APPLICANTS

CALIFORNIA INSTITUTE OF TECHNOLOGY, Pasadena, CA, UNITED STATES

INVENTORS

Emil P. KARTALOV, PASADENA, CA, UNITED STATES

Aditya RAJAGOPAL, ORANGE, CA, UNITED STATES

Axel SCHERER, BARNARD, VT, UNITED STATES

CONTINUING DATA

This application is a CON of 16937464 07/23/2020 PAT 11866768

16937464 is a CON of 15914356 03/07/2018 PAT 10770170

15914356 is a CON of 14451876 08/05/2014 PAT 10068051

14451876 is a CON of 13756760 02/01/2013 PAT 8838394

13756760 has PRO of 61703093 09/19/2012

13756760 has PRO of 61594480 02/03/2012

FOREIGN APPLICATIONS

IF REQUIRED, FOREIGN LICENSE GRANTED**

09/28/2023

**** SMALL ENTITY ****

STATE OR COUNTRY

UNITED STATES

ADDRESS

Sheppard, Mullin, Richter & Hampton LLP

650 Town Center Drive, 10th Floor

Costa Mesa, CA 92626

UNITED STATES

FILING FEE RECEIVED

$2,800

TO: SheppardMullin_Pair@firsttofile.com,docketing@sheppardmullin.com
FROM: noreply@uspto.gov
CC: patentcenter_eofficeaction@uspto.gov
SUBJECT: USPTO: Patent Electronic System - Correspondence Notification for Customer Number 30764

Mon Jul 08 05:01:27 EDT 2024

Dear Patent Center Customer:

Correspondence Address:

Sheppard, Mullin, Richter &Hampton LLP
650 Town Center Drive, 10th Floor
Costa Mesa,CALIFORNIA,92626
UNITED STATES

This is a courtesy notification regarding the following USPTO patent application(s) associated with your Customer Number, 30764, that have new outgoing correspondence. This correspondence is now available for viewing in Patent Center.

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Application	Document	Mailroom Date	Attorney Docket No.
18352112	NOA	07/08/2024	88DG-373535-US

To view your correspondence online, please sign in to Patent Center and then select Workbench/View correspondence. To update your email address(es), select Manage/Manage customer numbers.

If you have any questions, please contact the Patent Electronic Business Center (EBC) at ebc@uspto.gov or 866-217-9197 Monday – Friday, 6 a.m. to midnight ET.

Please do not reply to this email as it was sent from an unmonitored mailbox.

Sincerely,

The Patent Center Team

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Alexandria, VA 22313 - 1450
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ELECTRONIC PAYMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/352,112	06/07/2024 08:45:32 PM Z ET	88DG-373535-US

Title of Invention

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	3145	FILED BY	Annie Wong
PATENT CENTER #	65884283	AUTHORIZED BY	David Heisey
CUSTOMER #	30764	FILING DATE	07/13/2023
CORRESPONDENCE ADDRESS	-	FIRST NAMED INVENTOR	Emil P. KARTALOV

Payment Information

PAYMENT METHOD	PAYMENT TRANSACTION ID	PAYMENT AUTHORIZED BY
DA / 504561	E202467K46277029	David Heisey

FEE CODE	DESCRIPTION	ITEM PRICE($)	QUANTITY	ITEM TOTAL($)
2814	STATUTORY DISCLAIMER, INCLUDING TERMINAL DISCLAIMER	170.00	1	170.00
			TOTAL AMOUNT:	$170.00

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 filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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

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

New International Application Filed with the USPTO as a Receiving Office

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

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TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A PRIOR PATENT

APPLICATION #	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET #
18352112	07/13/2023	Emil KARTALOV	88DG-373535-US

Title of Invention

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

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

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

Owner	Percent Interest
CALIFORNIA INSTITUTE OF TECHNOLOGY	100%
Total	100%

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

Application #	Filing Date

as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and any patent granted on the reference application 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 owner 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 any patent granted on said reference application, "as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application," in the event that any such patent granted on the pending reference application: expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is 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 filed prior to its grant.

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

Patent #
8838394

as the term of said prior patent is presently shortened by any terminal disclaimer. The owner 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 owner does not disclaim the terminal part of the term 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 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 presently shortened by any terminal disclaimer.

☑ Terminal disclaimer fee under 37 CFR 1.20(d) included with Electronic Terminal Disclaimer request.

Applicant claims the following entity status:
Small

I hereby declare that all statements made herein of my own knowledge are true and that all statemnts made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I certify, in accordance with 37 CFR 1.4(d)(4) that I am: An attorney or agent registered to practice before the Patent and Trademark Office who is of record in this application

Signature	Name	Registration #
/David E. Heisey/	David Heisey	42651

* Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner). Form PTO/SB/96 may be used for making this certification. See MPEP 324.

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APPROVAL LETTER

APPLICATION #	FILING DATE	APPLICANT/PATENT UNDER REEXAMINATION
18/352,112	07/13/2023	Emil KARTALOV

Title of Invention

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Electronic terminal disclaimer filed on 06/07/2024

☑ Approved

This patent is subject to a Terminal Disclaimer

Approved / Disapproved by: Electronic Terminal Disclaimer automatically approved

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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/352,112	**06/07/2024 08:45:32 PM Z ET**	**88DG-373535-US**

Title of Invention

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

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CUSTOMER #	30764	FIRST NAMED INVENTOR	Emil P. KARTALOV
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	David Heisey

Documents

TOTAL DOCUMENTS: 2

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
petition-request.pdf	3	Terminal Disclaimer-Filed (Electronic)	48 KB
grantLetter.pdf	1	Terminal Disclaimer-Electronic-Approved	19 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
petition-request.pdf	C4D496807EF9B1CD7B9731D560442CCD903AD2E05B7720CB43073AA975373877DDCBA4C5CC25ACDB933468780A7EF377A

	A5BBDDB30462446163FB494DCAD22A4
grantLetter.pdf	BEF96A45F05EC89BE3ED03E4B9F6E8DC3C145E62EE46D519 5B870A5EE5DE7182FFB093D5AA2569DA4834EBD6394993236 8578BDFFA086249124FD87FB6D31D2F

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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 filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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

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

New International Application Filed with the USPTO as a Receiving Office

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

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/352,112	07/13/2023	Emil P. KARTALOV	88DG-373535-US	3145

30764 7590 06/07/2024

Sheppard, Mullin, Richter & Hampton LLP
650 Town Center Drive, 10th Floor
Costa Mesa, CA 92626

EXAMINER
NEGIN, RUSSELL SCOTT

ART UNIT	PAPER NUMBER
1672	

NOTIFICATION DATE	DELIVERY MODE
06/07/2024	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):

SheppardMullin_Pair@firsttofile.com
docketing@sheppardmullin.com

Advisory Action Before the Filing of an Appeal Brief	**Application No.** 18/352,112	**Applicant(s)** KARTALOV et al.	
	Examiner RUSSELL S NEGIN	**Art Unit** 1672	**AIA (FITF) Status** No

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

THE REPLY FILED 20 May 2024 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE.

NO NOTICE OF APPEAL FILED

1. ☑ The reply was filed after a final rejection. No Notice of Appeal has been filed. To avoid abandonment of this application, applicant must timely file one of the following replies: (1) an amendment, affidavit, or other evidence, which places the application in condition for allowance; (2) a Notice of Appeal (with appeal fee) in compliance with 37 CFR 41.31; or (3) a Request for Continued Examination (RCE) in compliance with 37 CFR 1.114 if this is a utility or plant application. Note that RCEs are not permitted in design applications. The reply must be filed within one of the following time periods:
   a) ☑ The period for reply expires 4 months from the mailing date of the final rejection.
   b) ☐ The period for reply expires on: (1) the mailing date of this Advisory Action; or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection.
   c) ☐ A prior Advisory Action was mailed more than 3 months after the mailing date of the final rejection in response to a first after-final reply filed within 2 months of the mailing date of the final rejection.The current period for reply expires ____ months from the mailing date of *the prior Advisory Action* or SIX MONTHS from the mailing date of the final rejection, whichever is earlier.
   Examiner Note: If box 1 is checked, check either box (a), (b) or (c). ONLY CHECK BOX (b) WHEN THIS ADVISORY ACTION IS THE FIRST RESPONSE TO APPLICANTS FIRST AFTER-FINAL REPLY WHICH WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. ONLY CHECK BOX (c) IN THE LIMITED SITUATION SET FORTH UNDER BOX (c). See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) or (c) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

NOTICE OF APPEAL

2. ☐ The Notice of Appeal was filed on _____. A brief in compliance with 37 CFR 41.37 must be filed within two months of the date of filing the Notice of Appeal (37 CFR 41.37(a)), or any extension thereof (37 CFR 41.37(e)), to avoid dismissal of the appeal. Since a Notice of Appeal has been filed, any reply must be filed within the time period set forth in 37CFR 41.37(a).

AMENDMENTS

3. ☑ The proposed amendments filed after a final rejection, but prior to the date of filing a brief, will not be entered because
   a) ☐ They raise new issues that would require further consideration and/or search (see NOTE below);
   b) ☐ They raise the issue of new matter (see NOTE below);
   c) ☑ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or
   d) ☐ They present additional claims without canceling a corresponding number of finally rejected claims.
   NOTE: The proposed amendments would raise new double patenting issues with US Patent 8,838,394 (See 37 CFR 1.116 and 41.33(a)).
4. ☐ The amendments are not in compliance with 37 CFR 1.121. See attached Notice of Non-Compliant Amendment (PTOL-324).
5. ☑ Applicant's reply has overcome the following rejection(s): See Continuation Sheet
6. ☐ Newly proposed or amended claim(s) _____ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).
7. ☑ For purposes of appeal, the proposed amendment(s):(a) ☑ will not be entered, or (b) ☐ will be entered, and an explanation of how the new or amended claims would be rejected is provided below or appended.

AFFIDAVIT OR OTHER EVIDENCE

8. ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____
9. ☐ The affidavit or other evidence filed after final action, but before or on the date of filing a Notice of Appeal will not be entered because applicant failed to provide a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented. See 37 CFR 1.116(e).
10. ☐ The affidavit or other evidence filed after the date of filing the Notice of Appeal, but prior to the date of filing a brief, will not be entered because the affidavit or other evidence failed to overcome all rejections under appeal and/or appellant fails to provide a showing of good and sufficient reasons why it is necessary and was not earlier presented. See 37 CFR 41.33(d)(1).
11. ☐ The affidavit or other evidence is entered. An explanation of the status of the claims after entry is below or attached.

REQUEST FOR RECONSIDERATION/OTHER

12. ☑ The request for reconsideration has been considered but does NOT place the application in condition for allowance because: See Continuation Sheet.
13. ☐ Note the attached Information *Disclosure Statement*(s). (PTO/SB/08) Paper No(s). _____
14. ☑ Other: PTO-413 interview summary is attached..

STATUS OF CLAIMS

15. The status of the claim(s) is (or will be) as follows:
    Claim(s) allowed:_____.
    Claim(s) objected to:4.
    Claim(s) rejected:1-3 and 5-30.
    Claim(s) withdrawn from consideration:_____.

/RUSSELL S NEGIN/ Primary Examiner, Art Unit 1672	

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Continuation of AMENDMENTS 5. Applicant's reply has overcome the following rejection(s): The proposed amendments would have overcome all of the rejections except the double patenting rejection with US Patent 8,838,394 had the proposed amendments been entered.

Continuation of REQUEST FOR RECONSIDERATION/OTHER 12. The request for reconsideration has been considered but does NOT place the application in condition for allowance because: The arguments are not persuasive because the arguments rely upon entry of the proposed amendments. Since the proposed amendments are not entered, the arguments are not persuasive. In addition, Sagner and Vogelstein do not require immobilization.

Examiner-Initiated Interview Summary	**Application No.** 18/352,112	**Applicant(s)** KARTALOV et al.		
	Examiner RUSSELL S NEGIN	**Art Unit** 1672	**AIA (First Inventor to File) Status** No	**Page** 1 of 1

All Participants (applicant, applicants representative, PTO personnel)	**Title**	**Type**
RUSSELL S NEGIN	Primary Examiner	Telephonic
David E. Heisey	Attorney	

Date of Interview: 28 May 2024

Issues Discussed:

Non-statutory Double Patenting

The Examiner left applicant's representative a voicemail offering an allowance if applicant files a Terminal Disclaimer with U.S. Patent 8,838,394. Applicant's representative has not retumed the voicemail.

/RUSSELL S NEGIN/ Primary Examiner, Art Unit 1672	

Applicant is reminded that a complete written statement as to the substance of the interview must be made of record in the application file. It is the applicants responsibility to provide the written statement, unless the interview was initiated by the Examiner and the Examiner has indicated that a written summary will be provided. See MPEP 713.04
Please further see:
MPEP 713.04
Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews, paragraph (b)
37 CFR § 1.2 Business to be transacted in writing

Applicant recordation instructions: 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.

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PATENT
Docket No.: 88DG-373535-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	)	
	)	Confirmation No.: 3145
Emil P. KARTALOV *et al.*	)	
	)	Examiner: Russell S. NEGIN
Application No.: 18/352,112	)	
	)	
Filed: July 13, 2023	)	Art Unit: 1672
	)	
For: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	)	***VIA Patent Center***

DO NOT ENTER
/RSN/ 6/3/2024

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

Commissioner:

RESPONSE TO FINAL OFFICE ACTION

In response to the final Office Action mailed February 12, 2024, the period for response ending on June 12, 2024, being submitted with payment for a one-month extension of time, Applicant respectfully requests the Examiner to reconsider this Application in light of the following.

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

Remarks begin on page 10 of this paper.

TO: SheppardMullin_Pair@firsttofile.com,docketing@sheppardmullin.com
FROM: noreply@uspto.gov
CC: patentcenter_eofficeaction@uspto.gov
SUBJECT: USPTO: Patent Electronic System - Correspondence Notification for Customer Number 30764

Fri Jun 07 05:01:39 EDT 2024

Dear Patent Center Customer:

Correspondence Address:

Sheppard, Mullin, Richter &Hampton LLP
650 Town Center Drive, 10th Floor
Costa Mesa,CALIFORNIA,92626
UNITED STATES

This is a courtesy notification regarding the following USPTO patent application(s) associated with your Customer Number, 30764, that have new outgoing correspondence. This correspondence is now available for viewing in Patent Center.

The official date of notification of the outgoing correspondence will be indicated on the form (e.g., PTOL-90) accompanying the correspondence.

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The list of documents shown below are provided as a courtesy and is not part of the official file wrapper. The content of the images shown in the Image File Wrapper is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
18352112	CTAV	06/07/2024	88DG-373535-US
18352112	EXIN	06/07/2024	88DG-373535-US

To view your correspondence online, please sign in to Patent Center and then select Workbench/View correspondence. To update your email address(es), select Manage/Manage customer numbers.

If you have any questions, please contact the Patent Electronic Business Center (EBC) at ebc@uspto.gov or 866-217-9197 Monday – Friday, 6 a.m. to midnight ET.

Please do not reply to this email as it was sent from an unmonitored mailbox.

Sincerely,

The Patent Center Team

UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
Alexandria, VA 22313 - 1450
www.uspto.gov

ELECTRONIC PAYMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/352,112	05/20/2024 12:43:16 PM Z ET	88DG-373535-US

Title of Invention

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	3145	FILED BY	Annie Wong
PATENT CENTER #	65592474	AUTHORIZED BY	David Heisey
CUSTOMER #	30764	FILING DATE	07/13/2023
CORRESPONDENCE ADDRESS	-	FIRST NAMED INVENTOR	Emil P. KARTALOV

Payment Information

PAYMENT METHOD	PAYMENT TRANSACTION ID	PAYMENT AUTHORIZED BY
DA / 504561	E20245JC44127203	Annie Wong

FEE CODE	DESCRIPTION	ITEM PRICE($)	QUANTITY	ITEM TOTAL($)
2251	EXTENSION FOR RESPONSE WITHIN FIRST MONTH	88.00	1	88.00
			TOTAL AMOUNT:	$88.00

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 filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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

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

New International Application Filed with the USPTO as a Receiving Office

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

PATENT
Docket No.: 88DG-373535-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	)	
	)	Confirmation No.: 3145
Emil P. KARTALOV *et al.*	)	
	)	Examiner: Russell S. NEGIN
Application No.: 18/352,112	)	
	)	
Filed: July 13, 2023	)	Art Unit: 1672
	)	
For: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	)	***VIA Patent Center***

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

Commissioner:

RESPONSE TO FINAL OFFICE ACTION

In response to the final Office Action mailed February 12, 2024, the period for response ending on June 12, 2024, being submitted with payment for a one-month extension of time, Applicant respectfully requests the Examiner to reconsider this Application in light of the following.

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

Remarks begin on page 10 of this paper.

AMENDMENTS TO THE CLAIMS:

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

1. (Currently Amended) A system comprising:

a sample chamber configured to house a sample and analyte-specific reagent mixtures of analyte-specific hybridization probes and multiple fluorophores;

a multi-channel detector to detect:

a first electromagnetic signal at a first wavelength from the sample chamber, the first electromagnetic signal generated by excitement of a first fluorophore of the multiple fluorophores;

a second electromagnetic signal at a second wavelength from the sample chamber, the second electromagnetic signal generated by excitement of a second fluorophore of the multiple fluorophores;

a third electromagnetic signal at a third wavelength from the sample chamber, the third electromagnetic signal generated by excitement of a third fluorophore of the multiple fluorophores;

a fourth electromagnetic signal at a fourth wavelength from the sample chamber, the fourth electromagnetic signal generated by excitement of a fourth fluorophore of the multiple fluorophores;

a processor controlled analyzer to receive, from the multi-channel detector, a cumulative signal based on the first, second, third, and fourth electromagnetic signals and apply a decoding matrix to the cumulative signal to unambiguously detect the presence or absence of at least each of M analytes by associating, for each analyte, a first value in a first component of the cumulative signal and a second value in a second component of the cumulative signal, wherein each first value is an intensity or range of intensities and each second value is a wavelength or a range of wavelengths, and wherein the second values comprise the first, second, third, and fourth wavelengths, and the determination is made without immobilization, mass spectrometry or melting curve analysis;

wherein for the positive integer M,

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M=C*log2 (F+1),

F is a positive integer and is equal to the maximum cumulative intensity of the first component of the signal, for any second value, when all of the analytes are present, and

C=4, 5, or 6; and

wherein F+1 is a positive integer and wherein F+1 is 1 or a power of 2.

wherein M is greater than the number of the second values used to encode the analytes (C), the multi-channel detector comprises C channels, and M and C are positive integers;.

2. (Currently Amended) The system of claim 1, wherein the multi-channel detector is further configured to detect:

a fifth electromagnetic signal at a fifth wavelength from the sample chamber, the fifth electromagnetic signal generated by excitement of a fifth fluorophore of the multiple fluorophores; ~~and~~

a sixth electromagnetic signal at a sixth wavelength from the sample chamber, the sixth electromagnetic signal generated by excitement of a sixth fluorophore of the multiple fluorophores; and

wherein C= 6.

3. (Currently Amended) The system of claim 1, ~~wherein for the positive integer M,~~

~~M=C*log2 (F+1),~~

~~F is a positive integer and is equal to the maximum cumulative intensity of the first component of the signal, for any second value, when all of the analytes are present, and~~

~~C=4, 5, or 6~~ wherein F=1, 3, 7, 15, 31, 63, or 127.

4. (Currently Amended) The system of claim [[3]]1, wherein F=3.

5. (Currently Amended) The system of claim [[1]]4, wherein the multiple fluorophores is selected from the group consisting of a blue fluorophore, a green fluorophore, a yellow fluorophore, a red fluorophore, and any combinations thereof.

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6. (Currently Amended) The system of claim [[1]]4, wherein the multiple fluorophores is selected from the group consisting of a fluorescein amidite (FAM), a cyanine 3 (Cy3), a carboxy-X-rhodamine (ROX), a cyanine 5 (Cy5), a cyanine 5.5 (Cy5.5), and any combinations thereof.

7. (Currently Amended) The system of claim [[1]]4, wherein the multiple fluorophores has a maximum excitation wavelength selected from the group consisting of about 494 nm, about 550 nm, about 567 nm, about 650 nm, about 675 nm, and any combinations thereof.

8. (Currently Amended) The system of claim [[1]]4, wherein the multiple fluorophores has a maximum emission wavelength selected from the group consisting of about 518 nm, about 565 nm, about 591 nm, about 670 nm, about 697 nm, and any combinations thereof.

9. (Currently Amended) The system of claim [[1]]4, wherein the analyte-specific hybridization probes are labeled with a fluorophore of the multiple fluorophores.

10. (Currently Amended) The system of claim [[1]]4, wherein the detector comprises four or six band pass filters.

11. (Currently Amended) The system of claim [[1]]4, wherein the detector comprises band pass filters that enable the detection of blue, green, and red fluorescent signals; and wherein the detector comprises band pass filters that enable the detection of ranges selected from the group consisting of from about 483 nm to about 533 nm, from about 523 nm to about 568 nm, from about 558 nm to about 610 nm, from about 615 nm to about 670 nm, and any combinations thereof.

12. (Currently Amended) The system of claim [[1]]4, wherein the multi-channel detector comprises four or six photodetectors.

13. (Currently Amended) The system of claim [[1]]4, wherein the multi-channel detector comprises photodetectors that enable the detection of blue, green, and red fluorescent signals.

14. (Currently Amended) The system of claim [[1]]4, wherein the multi-channel detector comprises photodetectors that enable the detection ranges selected from the group consisting of from about 483 nm to about 533 nm, from about 523 nm to about 568 nm, from about 558 nm to about 610 nm, from about 615 nm to about 670 nm, and any combinations thereof.

15. (Currently Amended) The system of claim [[1]]4, wherein the sample comprises a droplet.

16. (Previously Presented) The system of claim 15, wherein the droplet is housed in a dynamic array.

17. (Previously Presented) The system of claim 2, wherein each of the six fluorophores is attached to a hybridization probe.

18. (Currently Amended) The system of claim [[1]]4, further comprising a display coupled to the processor controlled analyzer to visualize a plot of the first and second values.

19. (Currently Amended) A system comprising:

a processor;

a display; and

a non-transitory computer readable medium storing instructions thereon that, when executed by the processor, cause the processor to:

obtain cumulative signal data from a digital PCR instrument with a sample volume, the digital PCR instrument comprising a light source and a multichannel detector with C channels, and the sample volume comprising M fluorescently labeled polynucleotide analytes;

apply a decoding matrix to the cumulative signal data to unambiguously determine the presence or absence of at least each of the M fluorescently labeled polynucleotide analytes by

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associating, for each fluorescently labeled polynucleotide analyte, a first value in a first component of the cumulative signal data and a second value in a second component of the cumulative signal data, wherein each first value is an intensity or range of intensities and each second value is a wavelength or a range of wavelengths,

wherein for a positive integer M,

$M=C*\log_2 (F+1)$,

F is the maximum cumulative intensity of the first component of the cumulative signal data, for any second value, when all of the analytes are present, ~~and~~

M, C, and F are each positive integers, and

wherein F+1 is a positive integer and wherein F+1 is 1 or a power of 2; and

plot, on the display, a representation of the first value and the second value;

wherein the cumulative signal data comprises:

a first electromagnetic signal at a first wavelength from the sample volume, the first electromagnetic signal generated by excitement of a first fluorescently labeled polynucleotide analyte of the six fluorescently labeled polynucleotide analytes;

a second electromagnetic signal at a second wavelength from the sample volume, the second electromagnetic signal generated by excitement of a second fluorescently labeled polynucleotide analyte of the six fluorescently labeled polynucleotide analytes;

a third electromagnetic signal at a third wavelength from the sample volume, the third electromagnetic signal generated by excitement of a third fluorescently labeled polynucleotide analyte of the six fluorescently labeled polynucleotide analytes;

a fourth electromagnetic signal at a fourth wavelength from the sample volume, the fourth electromagnetic signal generated by excitement of a fourth first fluorescently labeled polynucleotide analyte of the six fluorescently labeled polynucleotide analytes; and

wherein the second values comprise the first, second, third, and fourth wavelengths, and the determination is made without immobilization, mass spectrometry or melting curve analysis.

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20. (Currently Amended) A reaction mixture for unambiguously detecting a presence or absence of ~~a plurality of~~ M non-immobilized polynucleotide analytes of a single sample, the reaction mixture comprising:

a plurality of non-immobilized hybridization probes,

wherein the reaction mixture is subjected to conditions such that the plurality of polynucleotide analytes are amplified;

wherein at least one of the plurality of oligonucleotide primers is used to amplify a region complementary of at least one of the plurality of hybridization probes;

wherein the plurality of hybridization probes comprise a first hybridization probe conjugated to a first fluorophore and a second fluorophore,

wherein the first fluorophore and the second fluorophore, when excited by a light source, emit light of different wavelengths, and

the plurality of hybridization probes further comprises a third hybridization probe and a fourth hybridization probe, wherein the third hybridization probe is conjugated to a third fluorophore and the fourth hybridization is conjugated to a fourth fluorophore,

wherein the third fluorophore and the fourth fluorophore, when excited by a light source, emit light of a same wavelength, and

wherein the third hybridization probe and the fourth hybridization probe are present at different concentrations in the reaction mixture;

optionally the plurality of hybridization probes further comprises a fifth hybridization probe and a sixth hybridization probe, wherein the fifth hybridization probe is conjugated to a fifth fluorophore and the sixth hybridization is conjugated to a sixth fluorophore;

wherein the fifth fluorophore and the sixth fluorophore, when excited by a light source, emit light of a same wavelength, and

wherein the fifth hybridization probe and the six hybridization probe are present at different concentrations in the reaction mixture,

wherein for the positive integer M,

$M=C*\log_2(F+1)$,

F is a positive integer and is equal to the maximum cumulative intensity of the first component of the signal, for any second value, when all of the analytes are present, and

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C=4, 5, or 6; and

wherein F+1 is a positive integer and wherein F+1 is 1 or a power of 2, and

wherein M is greater than the number of the second values used to encode the analytes , the multi-channel detector comprises C channels, and M and C are positive integers.

21. (Currently Amended) ~~The reaction mixture of claim 20, wherein the reactions mixture is separated into droplets~~The reaction mixture of claim 20, wherein C=6 and wherein F=1, 3, 7, 15, 31, 63, or 127.

22. (Currently Amended) ~~The reaction mixture of claim 20, wherein the first fluorophore, the second fluorophore, the third fluorophore or the fourth fluorophore is selected from the group consisting of a fluorescein amidite (FAM), a cyanine 3 (CY3), a carboxy-X-rhodamine (ROX), a cyanine 5 (CY5), a cyanine 5.5 (CY5.5), and any combinations thereof~~ The reaction mixture of claim 20, wherein F=3.

23. (Currently Amended) ~~The reaction mixture of claim 20, wherein the first fluorophore, the second fluorophore, the third fluorophore or the fourth fluorophore has a maximum excitation wavelength selected from the group consisting of about 494 nm, about 550 nm, about 567 nm, about 650 nm, about 675 nm, and any combinations thereof~~ The system of Claim 19, wherein F=1, 3, 7, 15, 31, 63, or 127.

24. (Currently Amended) ~~The reaction mixture of claim 20, wherein the first fluorophore, the second fluorophore, the third fluorophore or the fourth fluorophore has a maximum emission wavelength selected from the group consisting of about 518 nm, about 565 nm, about 591 nm, about 670 nm, about 697 nm, and any combinations thereof~~ The system of Claim 19, wherein F=3.

25. (Currently Amended) The reaction mixture of claim [[20]]22, wherein a ratio of concentration between the first hybridization probe and the second hybridization probe is between 0.3 and 0.7 mol/mol.

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26. (Currently Amended) The reaction mixture of claim [[20]]22, wherein the reaction mixture further comprises a polymerase having 5′ to 3′ exonuclease activity and primers specific for the plurality of polynucleotide analytes.

27. (Currently Amended) The reaction mixture of claim [[24]]22, wherein the first fluorophore, the second fluorophore, the third fluorophore, the fourth fluorophore, the fifth fluorophore, or the sixth fluorophore comprise multiple fluorophores.

28. (Currently Amended) The reaction mixture of claim [[20]]22, wherein one of the plurality of hybridization probes further comprise a fluorescent quencher.

29. (Currently Amended) The reaction mixture of claim [[20]]22, wherein the first hybridization probe and the second hybridization probe are present at different concentrations in the reaction mixture.

30. (Currently Amended) A kit for the unambiguously detecting a presence or absence of a plurality of polynucleotide analytes of a single sample comprising the components claim [[20]]22 and instructions for the use of the kit.

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REMARKS

In the final Office Action, the Examiner:

I. rejects claims 3 and 19 under 35 U.S.C. § 112(b) as allegedly being indefinite, *Office Action* at 2;

II. rejects claims 3 and 19 under 35 U.S.C. § 112(a) as allegedly failing to comply with the written description requirement, *Office Action* at 4;

III. rejects claims 1-2, 5-14, 17-18, 20, 22-24, 27, and 29-30 under 35 U.S.C. § 103 as being rendered obvious by Han et al., Nature Biotechnology, volume 19, 2001, pages 631-635 (*Han*) and Published Application No. WO 2004/087950 A2 (*Sagner*), *Office Action* at 6;

IV. rejects claims 15-16, 21, 25-26, and 28 under 35 U.S.C. § 103 as being rendered obvious by *Han*, *Sagner*, and Vogelstein et al., PNAS, volume 96, 1999, pages 9236-9241 (*Vogelstein*), *Office Action* at 10;

V. rejects claim 19 for nonstatutory double patenting over claim 1 of U.S. Patent No. 8,838,394 B2 in view of *Sagner, Office Action* at 13;

VI. rejects claims 1 and 20 for nonstatutory double patenting over claim 1 of U.S. Patent No. 10,068,051 B2 in view of *Sagner, Office Action* at 14;

VII. rejects claims 1, 15, 20, and 21 for nonstatutory double patenting over claim 1 of U.S. Patent No. 10,770,170 B2 in view of *Sagner*; *Office Action* at 16, and

VIII. rejects claims 1 and 20 for nonstatutory double patenting over claim 22 of copending U.S. Application No. 16/937,464 in view of *Sagner, Office Action at* 16.

To expedite allowance of this Application, Applicant has made clarifying amendments to Claims 1-15 and 18-30. Independent Claims 1, 19, and 20 has been amended to recite "without immobilization," the formula of M, and that "F+1 is a positive integer and wherein F+1 is 1 or a power of 2." Support can be found at least at Table 8 and paragraph **[105]**.

Claims 5-15 and 18 have been amended to be dependent on Claim 4, which the Office Action deemed to contain allowable subject matter.

Claims 22 and 24 have been amended to recite the embodiment of Claim 4, which the Office Action deemed to contain allowable subject matter.

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Claims 25-30 have been amended to dependent on Claim 22, which has now been amended to recite the embodiment of Claim 4, which the Office Action deemed to contain allowable subject matter.

Claims 3, 21, and 23 has been amended to recite that wherein F=1, 3, 7, 15, 31, 63, or 127. Support can be found at least at Table 8 and paragraph **[105]**.

Claim 2 has been amended to recite C=6, an embodiment previously presented in Claim 3.

Claim 4 has been amended to be dependent on Claim 1, which has now been amended to incorporated the embodiments of Claim 3.

No new matter has been added.

Applicant respectfully requests reconsideration of this application, and the timely allowance of the pending claims for at least the following reasons.

I. The Rejection of Claims 3 and 19 under 35 U.S.C. § 112(b)

The Examiner rejects claims 3 and 19 under 35 U.S.C. § 112(b) as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor regards as the invention. In particular, the Examiner alleges that "[i]t is unclear as to the meets and bounds of M being an integer when F+1 [in the equation $M=C*\log_2(F+1)$] is not a power of 2" (Office Action at page 3). Applicant respectfully disagrees for at least the reasons previously submitted. Nevertheless, in the interest of an expedient allowance of this Application, Applicant has amended the claims to clarify that in the equation M=C*log2(F+1), "F+1 is a positive integer and wherein F+1 is 1 or a power of 2." See independent claims 1, 19 and 20. Support can be found at least at Table 8, which shows F=0, 1, 3, 7, and 15 corresponding to the F+1 as 0+1=1; 1+1=2; 3+1=4, 7+1=8, 15+1=16. Paragraph **[105]** further states:

The method presented above, and exemplified in Table 8, may be extended by introducing additional colors [C] and/or intensities….Additional intensity levels, such as [15+1=]16, [31+1=]32, [63+1=]64, [127+1=]128, [255+1=]255[sic, see paragraph **[106]**], [511+1=]512, [1023+1=]1024, [2047+1=]2048, [4095+1=]4096, [8191+1=]8192, [16383+1=]16384, [32767+1=]32768, [65535+1=]65536, and so on may also be used. The method is generalizable and may be used to encode an infinite number of analytes.

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Furthermore, claim 3 now further specifies that wherein F=1, 3, 7, 15, 31, 63, or 127. A person of ordinary skill in the art would have understood that log2(F+1), where F=1, 3, 7, 15, 31, 63, or 127, would result in an integer. Thus, reconsideration and withdrawal of the rejection are respectfully requested.

II. The Rejection of Claims 3 and 19 under 35 U.S.C. § 112(a)

The Examiner rejects claims 3 and 19 under 35 U.S.C. § 112(a) as allegedly to not enabling any person skilled in the art to make and use the invention. Applicant respectfully disagrees for the same reasons as Section I. Nevertheless, in the interest of an expedient allowance of this Application, Applicant has amended the claims. Reconsideration and withdrawal of the rejection are respectfully requested.

III. Rejection of Claims 1-2, 5-14, 17-18, 20, 22-24, 27, and 29-30 under 35 U.S.C. § 103

The Examiner rejects claims 1-2, 5-14, 17-18, 20, 22-24, 27, and 29-30 under 35 U.S.C. § 103 as being rendered obvious by *Han* and *Sagner*. Applicant respectfully disagrees for the same reasons previously submitted. Reconsideration and withdrawal of the rejection are respectfully requested.

A. Claims 4, 5-16, 18, 22, and 24-30

Additionally, Applicant notes that the Office Action deems Claim 4 to be allowable but for being dependent upon a rejected based claim. The Office Action at page 18 states: "[t]he prior art does not teach the equation in the claim, and mor specifically, the prior art does not teach the equation with the specific values of M, C, and F." Applicant respectfully submits that Applicant has amended the rejected base claim. Thus, Claim 4 should be allowable. Claim 5-16 and 18, as amended, depend directly or indirectly from Claim 4. Thus, Claims 5-16 and 18 should be allowable for at least the same reasons that Claim 4 is allowable. Claims 22 and 24, as amended embodies the same limitation as Claim 4. Thus, Claims 22 and 24 are allowable at least for the same reasons that Claim 4 is allowable. Claims 25-30 depends directly or indirectly from Claims 22 or 24. Thus, Claims 25-30 are allowable at least for the same reasons that Claims 22 and 24 are allowable.

B. Claims 1, 2, 17, 19-21 and 23

Additionally, Applicant submits that Claims 1, 19 and 20, as amended, is nonobvious over *Han* and *Sagner*, whether alone or in combination with another cited reference.

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First, Independent Claims 1 and 19 as amended, recites: "without immobilization." Independent Claim 20, recites "non-immobilized polynucleotide analytes." In stark contrast, *Han* teaches the ***immobilization of single-color signal beads*** on a polylysine-coated glass slide as expressly illustrated in Figure 2 (*Han*, at Figure 2, Page 632). Thus, it would not have been obvious for a person of ordinary skill in the art to combine *Han* with another cited reference to arrive at the claimed embodiments without undue experimentation. This is particularly so for *Sagner*, which does not use the quantum-dot-tagged microbeads technology. Claims 2, 17, 21 and 23 depend directly or indirectly from Claims 1, 19, and 20. Thus, Claims 2, 17, 21 and 23 are nonobvious for the same reasons that Claims 1, 19, and 20 are nonobvious.

Second, *Hans* and *Sagner*, whether alone or in combination with another cited reference fail to disclose the formula embodied in independent Claims 1, 19, and 20. Thus, it would not have been obvious for a person of ordinary skill in the art to combine *Han* with another cited reference to arrive at the claimed embodiments without undue experimentation. Claims 2, 17, 21 and 23 depend directly or indirectly from Claims 1, 19, and 20. Thus, Claims 2, 17, 21 and 23 are nonobvious for the same reasons that Claims 1, 19, and 20 are nonobvious.

Third, Claim 2 specifically recites C=6. In stark contrast, *Sagner* limits C at 5 or below. Thus, it would not have been obvious for a person of ordinary skill in the art to combine *Han* with another cited reference to arrive at the claimed embodiments without undue experimentation. Claim 17 depends directly or indirectly from Claim 2. Thus, Claim 17 is nonobvious for at least the same reason that Claim 2 is nonobvious.

For at least these reasons, reconsideration and withdrawal of the rejection are respectfully requested.

IV. Rejection of Claims 15-16, 21, 25-26, and 28 under 35 U.S.C. § 103

The Examiner rejects claims 15-16, 21, 25-26, and 28 under 35 U.S.C. § 103 as being rendered obvious by *Han*, *Sagner*, and *Vogelstein*. Applicant respectfully disagrees for the same reasons previously submitted. Reconsideration and withdrawal of the rejection are respectfully requested.

A. Claims 15-16, 25-26, and 28

Additionally, Applicant notes that the Office Action deems Claim 4 to is allowable but for being dependent upon a rejected based claim. Claims 15-16 as amended, depend directly or

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indirectly from Claim 4. Thus, Claims 15-16 should be allowable for at least the same reasons that Claim 4 is allowable. Claims 22 and 24, as amended embodies the same limitation as Claim 4. Thus, Claims 22 and 24 are allowable at least for the same reasons that Claim 4 is allowable. Claims 25-26 and 28 depends directly or indirectly from Claims 22 or 24. Thus, Claims 25-26, and 28 are allowable at least for the same reasons that Claims 22 and 24 are allowable.

B. Claim 21

Claim 21, as amended recites, [t]he reaction mixture of claim 20, wherein C=6 and F=1, 3, 7, 15, 31, 63, or 127." Claim 21 is nonobvious over the combination of *Han* and *Sagner* for the same reasons that Claim 20 is nonobvious over the combination of *Han* and *Sagner*, as discussed above. Additionally, Vogelstein describes a PCR method using two colors (MB-Red and MB-Green). See *Vogelstein* at Materials and Methods. Thus, reconsideration and withdrawal of the rejection are respectfully requested.

V. Nonstatutory Double Patenting

The Examiner further rejected the following claims of this Application alleging nonstatutory double patenting:

1. claim 19 for nonstatutory double patenting over claim 1 of U.S. Patent No. 8,838,394 B2 in view of *Sagner*;

2. claims 1 and 20 for nonstatutory double patenting over claim 1 of U.S. Patent No. 10,068,051 B2 in view of *Sagner*;

3. claim 1, 15, 20, and 21 for nonstatutory double patenting over claim 1 of U.S. Patent No. 10,770,170 B2 in view of *Sagner*; and

4. claims 1 and 20 for nonstatutory double patenting over claim 22 of copending U.S. Application No. 16/937,464 in view of *Sagner*.

Applicant requests that these matters be held in abeyance until such time as the claims of these applications are otherwise allowable. It is believed to be premature to file a Terminal Disclaimer before the scope of the claims has been settled.

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CONCLUSION

In view of the foregoing amendments and remarks, Applicant respectfully requests reconsideration of this application and the timely allowance of the pending claims.

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. Please grant any extensions of time required to enter this response and charge any additional required fees to Deposit Account No. 50-4561.

Respectfully submitted,

SHEPPARD MULLIN RICHTER & HAMPTON LLP

Dated: May 20, 2024

By: /David E. Heisey/
David E. Heisey
Reg. No. 42651
858.720.8900

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UNITED STATES
PATENT AND TRADEMARK OFFICE

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Alexandria, VA 22313 - 1450
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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/352,112	**05/20/2024 12:43:16 PM Z ET**	**88DG-373535-US**

Title of Invention

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	3145	FILED BY	Annie Wong
PATENT CENTER #	65592474	FILING DATE	07/13/2023
CUSTOMER #	30764	FIRST NAMED INVENTOR	Emil P. KARTALOV
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	David Heisey

Documents

TOTAL DOCUMENTS: 3

DOCUMENT		PAGES	DESCRIPTION	SIZE (KB)
FOA_Response_18352112.pdf		15	-	126 KB
FOA_Response_18352112-A.NE.pdf	(1-1)	1	Response After Final Action	55 KB
FOA_Response_18352112-CLM.pdf	(2-9)	8	Claims	50 KB
FOA_Response_18352112-REM.pdf	(10-15)	6	Applicant Arguments/Remarks Made in an Amendment	50 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
FOA_Response_18352112.pdf	63A5C2D6230A82F264416C006EFCE873A233DCFE73E819D9ED923F15EC7943DD69BF2E0A3AF8E2085546AF9E99CE76E330EED2CCD6203AC71F1D8D6E8E1AB678
FOA_Response_18352112-A.NE.pdf	188621DB2168696BC319A05196644F858248E373568427BAFEEF6B5424E9C44F8729FD1E984D4F85E4EB81B9F148B9FF6607D992680C3DD0E82D19EFF4B8AC28
FOA_Response_18352112-CLM.pdf	59FDC1D2D7CDC029BC061181655AD187179D228D442A78AD52C653FB86E173229DAA07B478318B43D808FF9FBB1FA2C4C3B50FEA16627A66FEC9A9C502F00B42
FOA_Response_18352112-REM.pdf	7F9FDE09B22786AF0A2116E6A3D50C1747C9C8709FD9431D8BEA7CEE6D5BEF5E70CD1B13090F7E1D77F32824E18F71FDFA481C786949AE8C724046CF2573ED1D

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 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 18/352,112	Filing Date 07/13/2023	☐ To be Mailed

ENTITY: ☐ LARGE ☑ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $40 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $ 192 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	05/20/2024	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 30	Minus	** 20	= 10		x $40 =	400
	Independent (37 CFR 1.16(h))	* 3	Minus	*** 3	= 0		x $ 192 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	400

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

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

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

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

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/352,112	07/13/2023	Emil P. KARTALOV	88DG-373535-US	3145

30764 7590 02/12/2024

SHEPPARD, MULLIN, RICHTER & HAMPTON LLP
650 Town Center Drive, 10th Floor
Costa Mesa, CA 92626

EXAMINER	
NEGIN, RUSSELL SCOTT	

ART UNIT	PAPER NUMBER
1672	

NOTIFICATION DATE	DELIVERY MODE
02/12/2024	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):

DOCKETING@SHEPPARDMULLIN.COM
SheppardMullin_Pair@firsttofile.com

Office Action Summary	Application No. 18/352,112	Applicant(s) KARTALOV et al.	
	Examiner RUSSELL S NEGIN	Art Unit 1672	AIA (FITF) 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 01 February 2024.

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

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

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

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

Disposition of Claims*

5) ☑ Claim(s) 1-30 is/are pending in the application.

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

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-3 and 5-30 is/are rejected.

8) ☑ Claim(s) 4 is/are objected to.

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

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

Application Papers

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

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

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

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

Priority under 35 U.S.C. § 119

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

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

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

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

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

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

Attachment(s)

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

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

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

4) ☐ Other: _____.

DETAILED ACTION

Comments

The present application is being examined under the pre-AIA first to invent provisions. 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 (i.e., changing from AIA to pre-AIA) 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.

Claims 1-30 are pending and examined in the instant Office action.

Information Disclosure Statements

The IDS filed on 2/1/2024 has been considered.

Claim Rejections - 35 USC § 112(b) - Indefiniteness

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.

The following rejection is reiterated:

Claims 3 and 19 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 applications subject to pre-AIA 35 U.S.C. 112, the applicant), regards as the invention.

Claims 3 and 19 recite the equation $M=C*log_2(F+1)$ wherein M is the number of analytes, C is the number of channels, and F is the number of fluorophores. It is unclear as to the meets and bounds of M being an integer when F+1 is not a power of 2.

Claim 20 is not rejected under this statute because when F=3, F+1 is 4 (a power of 2).

Response to arguments:

Applicant's arguments filed 1 February 2024 have been fully considered but they are not persuasive.

Applicant argues that one of ordinary skill in the art would know to choose F such that M is a positive integer. This argument is not persuasive because the claims only limit F to be a positive integer, and not a positive integer such that F+1 is a power of two. Amending the claims such that M is a positive integer does not limit F+1 to be a positive integer that is a power of two.

Claim Rejections - 35 USC § 112(a) - Scope of Enablement

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

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

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

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

The following rejection is reiterated:

Claims 3 and 19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification, while being enabling for providing a positive integer when F+1 is a power of 2, does not reasonably provide enablement for providing a positive integer when F+1 is not a power of 2. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

1. Claims 3 and 19 recite the equation $M=C*log_2(F+1)$ wherein M is the number of analytes, C is the number of channels, and F is the number of fluorophores. It is not understood as to how M is an integer when F+1 is not a power of 2.

Claim 20 is not rejected under this statute because when F=3, F+1 is 4 (a power of 2).

2. The specification is silent on how to determine M (a positive integer) when F+1 is not a power of 2.

3. The document of Han et al. [Nature Biotechnology, volume 19, 2001, pages 631-635; on IDS] teaches a system wherein each of the numbers of fluorophores, channels, and analytes are positive integers.

4. The claims recite an equation wherein the result (number of analytes) is not always a positive integer. The claims, specification, and prior art give no guidance on

how to determine the number of analytes when F+1 is not a power of 2. Consequently, it is unpredictable as to determining the number of analytes (as positive integer) when F+1 is not a power of 2. Such unpredictability yields UNDUE EXPERIMENTATION.

In view of the above, it is the Examiner's position that with the insufficient guidance and working examples and in view of unpredictability and the state of art, one of skill in the art could not make and/or use the invention with the claimed breadth without an undue amount of experimentation.

Response to arguments:

Applicant's arguments filed 1 February 2024 have been fully considered but they are not persuasive.

Applicant argues that one of ordinary skill in the art would know to choose F such that M is a positive integer. This argument is not persuasive because the claims only limit F to be a positive integer, and not a positive integer such that F+1 is a power of two. Amending the claims such that M is a positive integer does not limit F+1 to be a positive integer that is a power of two.

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject

matter pertains. Patentability shall not be negated by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

The following rejection is reiterated:

35 U.S.C. 103 Rejection #1:

Claims 1-2, 5-14, 17-18, 20, 22-24, 27, and 29-30 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Han et al. [Nature Biotechnology, volume 19, 2001, pages 631-635; on IDS] in view of Sagner et al. [WO 2004/087950 A2; on IDS].

Claim 1 is drawn to a system comprising a sample chamber configured to house a sample and analyte-specific reagent mixtures of analyte-specific hybridization probes and multiple fluorophores. The system comprises a multi-channel detector. This detector detects first, second, third, and fourth electromagnetic signals from first, second, third, and fourth wavelengths, respectively, from the sample chamber. The first, second, third, and fourth electromagnetic signals are generated by excitement of the first, second, third, and fourth fluorophores, respectively of the multiple fluorophores.

The system comprises a processor controlled analyzer to receive, from the multi-channel detector, a cumulative signal based on the electromagnetic signals and apply a decoding matrix to the cumulative signal to unambiguously detect the presence or absence of each of M analytes by associating, for each analyte, a first value in a first component of the cumulative signal and a second value in a second component of the cumulative signal. Each first value is an intensity or range of intensities, and each second value is a wavelength or range of wavelengths. The second values comprise the first, second, third, and fourth wavelengths, and the determination is made without mass spectrometry or melting curve analysis. The analysis requires that M is greater than the number of second values used to encode the analytes, the multi-channel detector comprises C channels, and M and C are positive integers.

Claim 2 is further limiting wherein the analysis is extended to fifth and sixth electromagnetic signals from fifth and sixth fluorophores.

Claim 20 is drawn to similar subject matter as claim 2, except claim 20 is drawn to a reaction mixture.

Claim 30 is drawn to similar subject matter as claim 20, except claim 30 is drawn to a kit.

The document of Han et al. studies quantum-dot-tagged microbeads for multiplexes optical coding of biomolecules [title]. Figure 1 on page 632 of Han et al. teaches that there are ten distinguishable colors of quantum dots that emit light at the ten listed wavelengths. Figures 5 and 6 of Han et al. use microbeads and hybridization to detect the presence of three of the quantum dot fluorophores. The spectral data of Figures 5 and 6 of Han et al. illustrates a matrix of intensity values of the fluorophores

as a function of wavelength of light. The number of analytes in Figure 1 of Han et al. (10) is greater than the numbers of second values in each of Figures 5 and 6 Han et al. (3).

Han et al. does not teach all of the channel/apparatus limitations of the claims.

The document of Sagner et al. studies an improved system for multicolor real-time PCR [title]. The cover figure of Sagner et al. illustrates the detection unit with six different detectors that measure signals at six different channels of wavelength. Page 9 of Sagner et al. lists the fluorophores. Page 10 of Sagner et al. lists wavelengths analyzed.

With regard to claim 5, Figures 5-6 of Han et al. illustrates blue, green, and red fluorophores.

With regard to claims 6 and 22, page 9 of Sagner et al. teaches use of the cyanine fluorophores.

With regard to claims 7-14, 17-18, 23-24, and 27, Figures 5-6 of Han et al. illustrates blue, green, and red fluorophores attached to hybridization probes. The spectral data of Figures 5-6 of Han et al. illustrate plots of the first and second values. Figure 1 of Han et al. lists the wavelengths of all ten possible quantum dots. The cover figure of Sagner et al. lists the wavelengths of each of the six channels/filters/photodetectors where blue, green, and red are a subset of the colors that are detected.

With regard to claim 29, Figures 5-6 of Han et al. illustrate different hybridization probes at different concentrations in a mixture.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the quantum dot analysis of Han et al. by use of the apparatus to analyze fluorophores of Sagner et al. wherein the motivation would have been that Sagner et al. teaches additional equipment to facilitate analysis of spectral data [cover figure of Sagner et al.]. There would have been a reasonable expectation of success to combine Han et al. and Sagner et al. because both studies are analogously applicable to analyzing spectral data relates to labeled biomolecules, either by quantum dots or fluorophores.

Response to arguments:

Applicant's arguments filed 1 February 2024 have been fully considered but they are not persuasive.

Applicant argues that the analysis of Han et al. does not apply to a cumulative signal. Absent a limiting description of the term “cumulative” in the disclosure, Han et al. is interpreted to measure the cumulative signal of a single bead.

Applicant argues that Han et al. involves immobilization of the particles. However, claims 1 and 19 do not exclude immobilization. In addition, in this obviousness prior art rejection, Sagner et al. does not involve immobilization.

The following rejection is reiterated:

35 U.S.C. 103 Rejection #2:

Claims 15-16, 21, 25-26, and 28 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Han et al. in view of Sagner et al. as applied to claims 1-2, 5-14, 17-18, 20, 22-24, 27, and 29-30 above, in further view of Vogelstein et al. [PNAS, volume 96, 1999, pages 9236-9241; on IDS].

Claims 15 and 21 are further limiting wherein the sample comprises a droplet. Claim 16 is further limiting wherein the droplets are housed in a dynamic array.

Claims 25-26 constrain ratios of probe concentrations and hybridization procedures.

Claim 28 is further limiting wherein a probe comprises a quencher.

Han et al. and Sagner et al. make obvious analyze of labeled biomolecules using a multichannel apparatus, as discussed above.

Han et al. and Sagner et al. do not teach the droplet, array, ratio of concentrations, quenchers, and hybridization procedures in the instantly rejected claims.

The document of Vogelstein et al. studies digital PCR [title]. Figure 1 on page 9237 of Vogelstein et al. illustrates droplets in a dynamic array and probe attached to a quencher. The Methods section on pages 9236-9237 of Vogelstein et al. teaches the hybridization procedures. A subset of the cells in Figure 5 on pages 9240 of Vogelstein et al. illustrates the recited ratios of hybridization probes.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the quantum dot analysis of Han et al. and the apparatus to analyze fluorophores of Sagner et al. by use of the digital PCR of Vogelstein et al. wherein the motivation would have been that Sagner et al. teaches additional empirical techniques to facilitate analysis of experimental data [Figure 1 of Vogelstein et al.]. There would have been a reasonable expectation of success to combine Han et al., Sagner et al., and Vogelstein et al. because all three studies are analogously applicable to analyzing spectral data relates to labeled biomolecules, either by quantum dots or fluorophores.

Response to arguments:

Applicant's arguments filed 1 February 2024 have been fully considered but they are not persuasive.

Applicant argue that the prior art does not teach four or more electromagnetic signals from four or more fluorophores. This argument is not persuasive because Figure 1 on page 632 of Han et al. teaches that there are ten distinguishable colors of quantum dots that emit light at the ten listed wavelengths.

Applicant reiterates the argument that the analysis of Han et al. does not apply to a cumulative signal. Absent a limiting description of the term “cumulative” in the disclosure, Han et al. is interpreted to measure the cumulative signal of a single bead. Assuming (*en arguendo*) that a cumulative signal relies on a plurality of signals from different particles, the analysis in Figure 5 of Vogelstein et al. relies on the cumulative signal of the relative combination of a red and green set of fluorophores.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 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); *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 nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP § 2146 *et seq.* 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 filing of a terminal disclaimer by itself is not a complete reply to a nonstatutory double patenting (NSDP) rejection. A complete reply requires that the terminal disclaimer be accompanied by a reply requesting reconsideration of the prior Office action. Even where the NSDP rejection is provisional the reply must be complete. See MPEP § 804, subsection I.B.1. For a reply to a non-final Office action, see 37 CFR 1.111(a). For a reply to final Office action, see 37 CFR 1.113(c). A request for reconsideration while not provided for in 37 CFR 1.113(c) may be filed after final for consideration. See MPEP §§ 706.07(e) and 714.13.

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

Double Patenting Rejection #1:

Claim 19 is rejected on the ground of nonstatutory double patenting as being unpatentable over claim 1 of U.S. Patent No. 8,838,394 B2 [on IDS] in view of Sagner et al.

Both claims are drawn to detecting the presence and absence of analytes using signals from fluorophores. However, the claim of '394 does not teach all of the apparatus limitations of the instantly rejected claim.

The document of Sagner et al. studies an improved system for multicolor real-time PCR [title]. The cover figure of Sagner et al. illustrates the detection unit with six different detectors that measure signals at six different channels of wavelength.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the fluorophore analysis of the claim of '394 by use of the apparatus to analyze fluorophores of Sagner et al. wherein the motivation would have been that Sagner et al. teaches additional equipment to facilitate analysis of spectral data [cover figure of Sagner et al.]. There would have been a reasonable expectation of success to combine the claim of '394 and Sagner et al. because both studies are analogously applicable to analyzing spectral data relates to fluorophore labeled biomolecules.

Response to arguments:

Applicant requests that the double patenting rejections be held in abeyance.

Double Patenting Rejection #2:

Claim [1 or 20] is rejected on the ground of nonstatutory double patenting as being unpatentable over claim 1 of U.S. Patent No. 10,068,051 B2 [on IDS] in view of Sagner et al.

Both sets of claims are drawn to detecting the presence and absence of analytes using signals from fluorophores. However, the claim of '051 does not teach all of the apparatus limitations of the instantly rejected claims.

The document of Sagner et al. studies an improved system for multicolor real-time PCR [title]. The cover figure of Sagner et al. illustrates the detection unit with six different detectors that measure signals at six different channels of wavelength.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the fluorophore analysis of the claim of '051 by use of the apparatus to analyze fluorophores of Sagner et al. wherein the motivation would have been that Sagner et al. teaches additional equipment to facilitate analysis of spectral data [cover figure of Sagner et al.]. There would have been a reasonable expectation of success to combine the claim of '051 and Sagner et al. because both studies are analogously applicable to analyzing spectral data relates to fluorophore labeled biomolecules.

Response to arguments:

Applicant requests that the double patenting rejections be held in abeyance.

Double Patenting Rejection #3:

Claims [1, 15, 20, or 21] are rejected on the ground of nonstatutory double patenting as being unpatentable over claim 1 of U.S. Patent No. 10,770,170 B2 [on IDS] in view of Sagner et al.

Both sets of claims are drawn to detecting the presence and absence of analytes using signals from fluorophores. However, the claim of '170 does not teach all of the apparatus limitations of the instantly rejected claims.

The document of Sagner et al. studies an improved system for multicolor real-time PCR [title]. The cover figure of Sagner et al. illustrates the detection unit with six different detectors that measure signals at six different channels of wavelength.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the fluorophore analysis of the claim of '170 by use of the apparatus to analyze fluorophores of Sagner et al. wherein the motivation would have been that Sagner et al. teaches additional equipment to facilitate analysis of spectral data [cover figure of Sagner et al.]. There would have been a reasonable expectation of success to combine the claim of '170 and Sagner et al. because both studies are analogously applicable to analyzing spectral data relates to fluorophore labeled biomolecules.

Response to arguments:

Applicant requests that the double patenting rejections be held in abeyance.

Double Patenting Rejection #4:

Claim [1 or 20] is provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claim 22 of copending Application No. 16/937,464 in view of Sagner et al.

Application '464 has been issued.

Both sets of claims are analogously drawn to detecting the presence and absence of analytes using signals from fluorophores. However, the claim of '464 does not teach all of the apparatus limitations of the instantly rejected claims.

The document of Sagner et al. studies an improved system for multicolor real-time PCR [title]. The cover figure of Sagner et al. illustrates the detection unit with six different detectors that measure signals at six different channels of wavelength.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the fluorophore analysis of the claim of '464 by use of the apparatus to analyze fluorophores of Sagner et al. wherein the motivation would have been that Sagner et al. teaches additional equipment to facilitate analysis of spectral data [cover figure of Sagner et al.]. There would have been a reasonable expectation of success to combine the claim of '464 and Sagner et al. because both studies are analogously applicable to analyzing spectral data relates to fluorophore labeled biomolecules.

Response to arguments:

Applicant requests that the double patenting rejections be held in abeyance.

Allowable Subject Matter

Claim 4 is objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

The prior art does not teach the equation in the claim, and more specifically, the prior art does not teach the equation with the specific values of M, C, and F.

E-mail Communications Authorization

Per updated USPTO Internet usage policies, Applicant and/or applicant's representative is encouraged to authorize the USPTO examiner to discuss any subject matter concerning the above application via Internet e-mail communications. See MPEP 502.03. To approve such communications, Applicant must provide written authorization for e-mail communication by submitting the following statement via EFS-Web (using PTO/SB/439) or Central Fax (571-273-8300):

> Recognizing that Internet communications are not secure, I hereby authorize the USPTO to communicate with the undersigned and practitioners in accordance with 37 CFR 1.33 and 37 CFR 1.34 concerning any subject matter of this application by video conferencing, instant messaging, or electronic mail. I understand that a copy of these communications will be made of record in the application file.

Written authorizations submitted to the Examiner via e-mail are **NOT** proper. Written authorizations must be submitted **via EFS-Web** (using PTO/SB/439) **or Central Fax** (571-273-8300). A paper copy of e-mail correspondence will be placed in the patent application when appropriate. E-mails from the USPTO are for the sole use of the intended recipient, and may contain information subject to the confidentiality requirement set forth in 35 USC § 122. See also MPEP 502.03.

Conclusion

No claim is allowed.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the Examiner should be directed to Russell Negin, whose telephone number is (571) 272-1083. This Examiner can normally be reached from Monday through Thursday from 8 am to 3 pm and variable hours on Fridays.

If attempts to reach the Examiner by telephone are unsuccessful, the Examiner's Supervisor, Larry Riggs, Supervisory Patent Examiner, can be reached at (571) 270-3062.

/RUSSELL S NEGIN/
Primary Examiner, Art Unit 1672
3 February 2024

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/352,112	KARTALOV et al.
	Examiner	Art Unit
	RUSSELL S NEGIN	1672

CPC - Searched*		
Symbol	Date	Examiner
C12Q1/6825 or C12Q1/68 or C12Q1/6851 or G01N21/6486 or G16B5/00 or G16B25/00 or G16B25/20 or G16B40/10 or G06F17/10 or G06F17/11 or G16B40/00	02/03/2024	RSN

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

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

Search Notes		
Search Notes	Date	Examiner
see attached search history printouts	02/03/2024	RSN
PE2E Search prior art search	02/03/2024	RSN
PE2E Search inventor search on all inventors	02/03/2024	RSN
PDP inventor search	02/03/2024	RSN
PE2E Search CPC code search with modified terms	02/03/2024	RSN

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

/RUSSELL S NEGIN/ Primary Examiner, Art Unit 1672	

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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/352,112	KARTALOV et al.
	Examiner	Art Unit
	RUSSELL S NEGIN	1672

/RUSSELL S NEGIN/ Primary Examiner, Art Unit 1672	

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

PTO/SB/08a (01-10)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	18352112
Filing Date	2023-07-13
First Named Inventor	Emil P. KARTALOV
Art Unit	1672
Examiner Name	Russell Scott NEGIN
Attorney Docket Number	88DG-373535-US

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
/RSN/	1	20090297525	A1	2009-12-03	DePinho et al.	

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/RSN/	1	102150043	CN	A	2011-08-10	Nat. Inst. for Medical Res.		☒
/RSN/	2	104271767	CN	A	2015-01-07	California Institute of Technology CalTech		☒
/RSN/	3	107326085	CN	A	2017-11-07	California Institute of Technology CalTech		☒

/RUSSELL S NEGIN/ 02/03/2024

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	18352112
	Filing Date	2023-07-13
	First Named Inventor	Emil P. KARTALOV
	Art Unit	1672
	Examiner Name	Russell Scott NEGIN
	Attorney Docket Number	88DG-373535-US

/RSN/	4	2008/005248	WO	A2	2008-01-10	Canon U.S. Life Sciences, Inc.		☐

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
/RSN/	1	First Search dated January 5, 2024, issued in related Chinese Application No. 202210389347.4 (3 pages).	☐
/RSN/	2	Dongyue LÜ et al., "Development of a xMAP liquid chip assay for the rapid identification of 7 common foodborne pathogens and its application", Journal of Hygiene Research, Vol. 41, No. 1, January 2012, with English language abstract.	☒

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EXAMINER SIGNATURE

Examiner Signature	/RUSSELL S NEGIN/	Date Considered	02/03/2024

*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.

18352112
Google Scholar Search
/RSN/ 2/3/2024

fluorophore cumulative signal
fluorophore cumulative signal wavelengths
fluorophore cumulative signal wavelengths channels
fluorophore cumulative signal wavelengths channels decoding
fluorophore cumulative signal wavelengths channels decoding intensities
fluorophore cumulative signal wavelengths channels decoding electromagnetic
fluorophore cumulative signal wavelengths channels decoding analytes

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PE2E SEARCH - Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp
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L2	0	"9376464".ap.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2023/12/30 02:26 PM
L3	16	"937464".ap.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2023/12/30 02:26 PM
L4	164	("10066263" OR "10068051" OR "10301668" OR "10770170" OR "20020022273" OR "20020146734" OR "20030134320" OR "20030148280" OR "20030148544" OR "20040053230" OR "20040191794" OR "20040248082" OR "20050053950" OR "20050064435" OR "20050106607" OR "20050164264" OR "20050214753" OR "20050250146" OR "20050260640" OR "20060039918" OR "20060216708" OR "20070072211" OR "20070178485" OR "20070231824" OR "20080050737" OR "20080069733" OR "20080096767" OR "20080124705" OR "20090042735" OR "20090048785" OR "20090062129" OR "20100015607" OR "20100041092" OR "20100047924" OR "20100112710" OR "20100120043" OR "20100151443" OR "20100159447" OR "20100210472" OR "20100233686" OR "20100248257" OR "20100261026" OR "20100267064" OR	(US-PGPUB; USPAT)	OR	ON	ON	2023/12/30 02:40 PM

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		"9422593" OR "9441266" OR "9447457" OR "9458497" OR "9791372").pn.					
L5	147	kartalov.in. NEAR emil.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)	OR	ON	ON	2023/12/30 02:42 PM
L6	299	rajagopal.in. NEAR aditya.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)	OR	ON	ON	2023/12/30 02:42 PM
L7	1102	scherer.in. NEAR axel.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)	OR	ON	ON	2023/12/30 02:42 PM
L8	245314	(C12Q1/6825 or C12Q1/68 or C12Q1/6851 or G01N21/6486 or G16B5/00 or G16B25/00 or	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB,	OR	ON	ON	2023/12/30 02:43 PM

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		G16B25/20 or G16B40/10 or G06F17/10 or G06F17/11 or G16B40/00).cpc.	HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
L9	1302	L5 L6 L7	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:43 PM
L10	124	L8 AND L9	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:43 PM
L11	669450	analytes	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT;	OR	ON	ON	2023/12/30 02:44 PM

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			IBM_TDB)				
L12	74	L10 AND L11	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:44 PM
L13	24385663	channels	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:44 PM
L14	49	L12 AND L13	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:44 PM
L15	7800095	wavelength	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS,	OR	ON	ON	2023/12/30 02:44 PM

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			IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
L16	37838	L15 AND L8	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:44 PM
L17	69	L9 AND L16	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:44 PM
L18	368464	fluorophores	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:44 PM

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L19	66	L17 AND L18	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:44 PM
L20	9645392	detector	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:45 PM
L21	53	L19 AND L20	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2023/12/30 02:45 PM
L22	1	("11009486").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2023/12/31 07:15 AM
L23	1	("20110264420").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2023/12/31 02:49 PM
L24	4	("8838394," "10068051," "10770170," "11866768").pn.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/02/03 11:31 AM

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L25	0	"9376464".ap.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/02/03 11:31 AM
L26	17	"937464".ap.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2024/02/03 11:31 AM
L27	164	("10066263" OR "10068051" OR "10301668" OR "10770170" OR "20020022273" OR "20020146734" OR "20030134320" OR "20030148280" OR "20030148544" OR "20040053230" OR "20040191794" OR "20040248082" OR "20050053950" OR "20050064435" OR "20050106607" OR "20050164264" OR "20050214753" OR "20050250146" OR "20050260640" OR "20060039918" OR "20060216708" OR "20070072211" OR "20070178485" OR "20070231824" OR "20080050737" OR "20080069733" OR "20080096767" OR "20080124705" OR "20090042735" OR "20090048785" OR "20090062129" OR "20100015607" OR "20100041092" OR "20100047924" OR "20100112710" OR "20100120043" OR "20100151443" OR "20100159447" OR "20100210472" OR "20100233686" OR "20100248257" OR "20100261026" OR "20100267064" OR "20100273173" OR "20100317005" OR "20100324834" OR "20110104684" OR "20110151459" OR "20110151550" OR "20110171658" OR "20110183884" OR "20110207623" OR "20110223602" OR	(US-PGPUB; USPAT)	OR	ON	ON	2024/02/03 11:31 AM

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		"20110237459" OR "20120003646" OR "20120040349" OR "20120040352" OR "20120045756" OR "20120077195" OR "20120101740" OR "20120122704" OR "20120141995" OR "20120171677" OR "20120184017" OR "20120190030" OR "20120196283" OR "20120244534" OR "20120252014" OR "20120252017" OR "20120258457" OR "20120302448" OR "20130017971" OR "20130040841" OR "20130078626" OR "20130116780" OR "20130261019" OR "20130288244" OR "20140004520" OR "20140038195" OR "20140171341" OR "20140213471" OR "20140274774" OR "20150057178" OR "20150140554" OR "20150211054" OR "20150275295" OR "20160040256" OR "20160108464" OR "20160201122" OR "20160273048" OR "20170145490" OR "20170314073" OR "20170362636" OR "20180030551" OR "20180052110" OR "20180057864" OR "20190002963" OR "20190032112" OR "20190112636" OR "20190233882" OR "20200010876" OR "5079351" OR "5538848" OR "5618711" OR "5677152" OR "5723591" OR "5773258" OR "5789224" OR "5804375" OR "5834203" OR "5876930" OR					

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		"5882856" OR "5928862" OR "5948360" OR "5981180" OR "5994056" OR "6030787" OR "6103463" OR "6127155" OR "6171785" OR "6258569" OR "6534266" OR "6534274" OR "6548259" OR "6642062" OR "6893875" OR "7101663" OR "7141377" OR "7348141" OR "7385043" OR "7410764" OR "7413708" OR "7473767" OR "7507575" OR "7575864" OR "7667024" OR "7671184" OR "7709249" OR "7767423" OR "7771949" OR "7919237" OR "7919244" OR "7930106" OR "7941279" OR "8039215" OR "8148512" OR "8426132" OR "8455184" OR "8492094" OR "8519115" OR "8614061" OR "8771955" OR "8838394" OR "8877464" OR "8962250" OR "9133506" OR "9222128" OR "9260761" OR "9366632" OR "9422593" OR "9441266" OR "9447457" OR "9458497" OR "9791372").pn.					
L28	151	kartalov.in. NEAR emil.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU,	OR	ON	ON	2024/02/03 11:31 AM

			CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)				
L29	304	rajagopal.in. NEAR aditya.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)	OR	ON	ON	2024/02/03 11:31 AM
L30	1105	scherer.in. NEAR axel.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)	OR	ON	ON	2024/02/03 11:31 AM
L31	246800	(C12Q1/6825 or C12Q1/68 or C12Q1/6851 or G01N21/6486 or G16B5/00 or G16B25/00 or G16B25/20 or G16B40/10 or G06F17/10 or G06F17/11 or G16B40/00).cpc.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM

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L32	1309	L5 L6 L7	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM
L33	127	L8 AND L9	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM
L34	674085	analytes	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM
L35	77	L10 AND L11	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL,	OR	ON	ON	2024/02/03 11:31 AM

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			NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
L36	24551447	channels	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM
L37	52	L12 AND L13	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM
L38	7848544	wavelength	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM
L39	38061	L15 AND L8	(US-PGPUB; USPAT;	OR	ON	ON	2024/02/03

			USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)				11:31 AM
L40	72	L9 AND L16	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM
L41	371430	fluorophores	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2024/02/03 11:31 AM
L42	69	L17 AND L18	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL,	OR	ON	ON	2024/02/03 11:31 AM

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			PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
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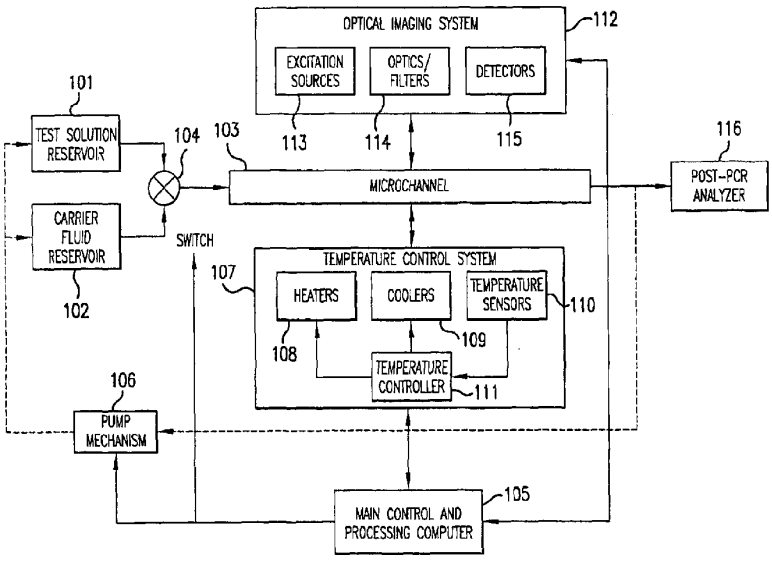
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— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

[Continued on next page]

(54) **Title:** REAL-TIME PCR IN MICRO-CHANNELS



(57) **Abstract:** The present invention relates to methods for amplifying nucleic acids in micro-channels. More specifically, the present invention relates to methods for performing a real-time polymerase chain reaction (PCR) in a continuous-flow microfluidic system and to methods for monitoring real-time PCR in such systems.

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REAL-TIME PCR IN MICRO-CHANNELS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] The present application claims priority to U.S. provisional patent application Serial No. 60/806,440 filed, June 30, 2006, which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

[0002] The present invention relates to methods for amplifying nucleic acids in micro-channels. More specifically, the present invention relates to methods for performing a real-time polymerase chain reaction (PCR) in a continuous-flow microfluidic system and to methods for monitoring real-time PCR in such systems.

2. Description of Related Art

[0003] The detection of nucleic acids is central to medicine, forensic science, industrial processing, crop and animal breeding, and many other fields. The ability to detect disease conditions (e.g., cancer), infectious organisms (e.g., HIV), genetic lineage, genetic markers, and the like, is ubiquitous technology for disease diagnosis and prognosis, marker assisted selection, correct identification of crime scene features, the ability to propagate industrial organisms and many other techniques. Determination of the integrity of a nucleic acid of interest can be relevant to the pathology of an infection or cancer. One of the most powerful and basic technologies to detect small quantities of nucleic acids is to replicate some or all of a nucleic acid sequence many times, and then analyze the amplification products. PCR is perhaps the most well-known of a number of different amplification techniques.

[0004] PCR is a powerful technique for amplifying short sections of DNA. With PCR, one can quickly produce millions of copies of DNA starting from a single template DNA molecule.

PCR includes a three phase temperature cycle of denaturation of DNA into single strands, annealing of primers to the denatured strands, and extension of the primers by a thermostable DNA polymerase enzyme. This cycle is repeated so that there are enough copies to be detected and analyzed. In principle, each cycle of PCR could double the number of copies. In practice, the multiplication achieved after each cycle is always less than 2. Furthermore, as PCR cycling continues, the buildup of amplified DNA products eventually ceases as the concentrations of required reactants diminish. For general details concerning PCR, see Sambrook and Russell, *Molecular Cloning -- A Laboratory Manual* (3rd Ed.), Vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (2000); *Current Protocols in Molecular Biology*, F. M. Ausubel et al., eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (supplemented through 2005) and *PCR Protocols A Guide to Methods and Applications* , M.A. Innis et al., eds., Academic Press Inc. San Diego, Calif. (1990).

[0005] Real-time PCR refers to a growing set of techniques in which one measures the buildup of amplified DNA products as the reaction progresses, typically once per PCR cycle. Monitoring the accumulation of products over time allows one to determine the efficiency of the reaction, as well as to estimate the initial concentration of DNA template molecules. For general details concerning real-time PCR see *Real-Time PCR: An Essential Guide*, K. Edwards et al., eds., Horizon Bioscience, Norwich, U.K. (2004).

[0006] Several different real-time detection chemistries now exist to indicate the presence of amplified DNA. Most of these depend upon fluorescence indicators that change properties as a result of the PCR process. Among these detection chemistries are DNA binding dyes (such as SYBR® Green) that increase fluorescence efficiency upon binding to double stranded DNA. Other real-time detection chemistries utilize Foerster resonance energy transfer (FRET), a

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phenomenon by which the fluorescence efficiency of a dye is strongly dependent on its proximity to another light absorbing moiety or quencher. These dyes and quenchers are typically attached to a DNA sequence-specific probe or primer. Among the FRET-based detection chemistries are hydrolysis probes and conformation probes. Hydrolysis probes (such as the TaqMan® probe) use the polymerase enzyme to cleave a reporter dye molecule from a quencher dye molecule attached to an oligonucleotide probe. Conformation probes (such as molecular beacons) utilize a dye attached to an oligonucleotide, whose fluorescence emission changes upon the conformational change of the oligonucleotide hybridizing to the target DNA.

[0007] A number of commercial instruments exist that perform real-time PCR. Examples of available instruments include the Applied Biosystems PRISM 7500, the Bio-Rad iCylcer, and the Roche Diagnostics LightCycler 2.0. The sample containers for these instruments are closed tubes which typically require at least a 10 µl volume of sample solution. If the lowest concentrations of template DNA detectable by a particular assay were on the order of one molecule per microliter, the detection limit for available instruments would be on the order of tens of targets per sample tube. Therefore, in order to achieve single molecule sensitivity, it is desirable to test smaller sample volumes, in the range of 1-1000 nl.

[0008] More recently, a number of high throughput approaches to performing PCR and other amplification reactions have been developed, e.g., involving amplification reactions in microfluidic devices, as well as methods for detecting and analyzing amplified nucleic acids in or on the devices. Thermal cycling of the sample for amplification is usually accomplished in one of two methods. In the first method, the sample solution is loaded into the device and the temperature is cycled in time, much like a conventional PCR instrument. In the second method, the sample solution is pumped continuously through spatially varying temperature zones.

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[0009] For example, Lagally et al. (*Anal Chem* **73**:565-570 (2001)) demonstrated amplification and detection of single template DNA in a 280 nl PCR chamber. Detection of products was made post-PCR using capillary electrophoresis. On the other hand, Kopp et al. (*Science* **280**:1046-1048 (1998)) demonstrated continuous-flow PCR using a glass substrate with a serpentine channel passing over three constant temperature zones at 95° C (denature), 72° C (extension), and 60° C (annealing). The 72° C zone was located in the central region and had to be passed through briefly in going from 95° C to 60° C. Detection was made post-PCR using gel electrophoresis. Since this PCR technique is not based on heating the entire surfaces of the reaction vessel, the reaction rate is determined by a flow rate, not a heating/cooling rate. Neither of these references described real-time monitoring of the PCR reaction.

[0010] Park et al. (*Anal Chem* **75**:6029-6033 (2003)) describe a continuous-flow PCR device that uses a polyimide coated fused silica capillary wrapped into a helix around three temperature-controlled blocks. Sample volumes were 2 μl. Detection was made post PCR using gel electrophoresis. Reference was made to the possibility of adapting their instrument for real-time PCR by using a capillary coated with PTFE instead of non-transparent polyimide. See also, Hahn et al. (WO 2005/075683).

[0011] Enzelberger et al. (U.S. Patent No. 6,960,437) describe a microfluidic device that includes a rotary channel having three temperature zones. A number of integrated valves and pumps are used to introduce the sample and to pump it through the zones in a rotary fashion.

[0012] Knapp et al. (U.S. Patent Application Publication No. 2005/0042639) describe a microfluidic device capable of single molecule amplification. A planar glass chip with several straight parallel channels is disclosed. A mixture of target DNA and PCR reagents is injected into these channels. In a first embodiment, the channels are filled with this mixture and flow is

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stopped. Then the entire length of the channels is thermally cycled. After thermal cycling is completed, the channels are imaged in order to detect regions of fluorescence where DNA has been amplified. In a second embodiment, the PCR mixture flows continuously through the amplification zone as the temperature is cycled, and fluorescence is detected downstream of the amplification zone. Different degrees of amplification are achieved by altering the time spent in cycling, through changing distance traveled under cycling, and the like. It is worth noting that this method varies conditions (such as cycles experienced) for separate consecutive sample elements, rather than monitoring the progress of individual sample elements over time.

[0013] None of this art has combined single target molecule sensitivity with real-time reaction monitoring capability in a continuous flow device. Thus, a need exists for robust high throughput methods of real-time PCR that can be performed efficiently and accurately with small sample volumes. It would be desirable that the methods have single target molecule sensitivity, use lower quantities of PCR reagents and use less energy for temperature cycling. The present invention provides these and other features that will be apparent upon review of the description of the invention.

SUMMARY OF THE INVENTION

[0014] The present invention relates to methods for amplifying nucleic acids in micro-channels. More specifically, the present invention relates to methods for performing a real-time polymerase chain reaction (PCR) in a continuous-flow microfluidic system and to methods for monitoring real-time PCR in such systems.

[0015] Thus, in a first aspect, the present invention provides a method of performing real-time PCR comprising the steps of: a) continuously moving a bolus of test solution containing real-

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time PCR reagents in a channel; b) moving a carrier-fluid in the channel, sequentially alternating with a test bolus; c) cycling the temperature in a defined section of the channel in order to achieve PCR; and d) measuring the intensity of the fluorescent signal at a plurality of locations along the defined section of the channel. In one embodiment, the channel is a micro-channel or a microfluidic channel, such that the dimensions of the channel are small enough to allow for the amplification and detection of a single DNA molecule originally present in the test solution. In another embodiment, the test solution is substantially the same as the carrier fluid, except that it comprises all the necessary real-time PCR reagents. The real-time PCR reagent mixture may include PCR primers, dNTPs, polymerase enzymes, salts, buffers, surface-passivating agents, and the like. In addition, the real-time PCR mixture may include a non-specific fluorescent DNA detecting molecule, a sequence-specific fluorescent DNA probe or a marker. In an additional embodiment, the carrier fluid is an immiscible fluid. The purpose of the carrier fluid is to deter transfer of material from one test bolus to another. Another purpose of the carrier fluid is to provide a distinguishable transition between boluses that may be used to track the fluid flow in the channel. The carrier fluid may include a marker.

[0016] In one embodiment, the temperature is cycled using a thermal transfer element along the defined section of the channel. In another embodiment, the thermal transfer element cycles the temperature in the entire defined section of the channel. That is, a constant temperature zone is used to provide the thermal cycling. In a further embodiment, an appropriately programmed computer controls the temperature cycling of the thermal transfer element. In one embodiment, the temperature of the thermal transfer element is detected and fed back to the computer. In accordance with these embodiments, heating and cooling are applied to a length of the channel (the reaction zone) such that its temperature follows a PCR profile in time. The test boluses are

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pumped through this reaction zone at a flow rate (speed) such that the number of PCR cycles required is achieved during the time the bolus flows from the upstream end to the downstream end of the reaction zone.

[0017] In another embodiment, the intensity of the fluorescent signal is measured at a specific time and/or temperature during the PCR temperature cycle. In an additional embodiment, the intensity of the fluorescent signal is measured once during each PCR cycle. In a further embodiment, the plurality of locations at which the intensity of the fluorescent signal is measured is the entire defined section of the channel. In another embodiment, the data concerning the intensity of the fluorescent signal is processed by an appropriately programmed computer. In another embodiment, an image of at least one fluorescent signal along the length of the channel is made. In a further embodiment, the image capture is performed repeatedly on consecutive temperature cycling periods. The image may be created or the intensity of the fluorescent signal measured using a multiple-pixel array detector. A stationary mechanism or a scanning mechanism or both may be used to capture the image.

[0018] In a second aspect, the present invention provides a method for performing real-time PCR that includes monitoring the average flow speed of the liquid in a channel. In accordance with this aspect, the method comprises the steps as set forth in the first aspect and further comprises the step of: e) measuring the average flow speed of the fluid in the channel. By monitoring the flow speed in the channel, the location of a test bolus as a function of time can be determined, thus identifying the number of PCR cycles that has been experienced by the test bolus.

[0019] In one embodiment, average flow speed of the fluid is measured by comparing sequential images of the reaction-dependent fluorescent signal from the channel. In a second

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embodiment, the average flow speed of the fluid is measured by comparing sequential images of a marker (i.e., a reaction independent marker) in the channel. The use of the marker has the advantage that if the real-time PCR signal is not detectable, the fluid flow would still be detectable. Examples of potential markers are dyes, semiconductor quantum dots, polymer microbeads, scattering metal particles, microbubbles, and the like known to skilled artisans. In one embodiment, the marker is present in the test solution. If the marker is present in the test solution, it should not adversely affect any PCR chemical reactions. In another embodiment, the marker is present in the carrier fluid. In a further embodiment, the marker is a separate bolus in series with test boluses. In an additional embodiment, the marker is resolvable from the fluorescent signal, such as by excitation wavelength, emission spectrum, lifetime, and the like well known in the art. In a further embodiment, the data concerning the sequential images of the intensity of the fluorescent signal or of the marker is processed by an appropriately programmed computer. In accordance with these embodiments, the average flow speed is determined by comparing images taken from consecutive PCR cycles. In a still further embodiment, the flow speed can be measured using a reaction zone entrance detector and a volume flow rate meter. Flow speed could be estimated, for example, by knowing the dimensions of the channel and measuring the volume flow rate.

[0020] In a third aspect, the present invention provides a method for performing real-time PCR that includes monitoring the flow speed of the liquid in a channel and adjusting the flow speed as necessary to control the duration of the PCR cycling experienced by a test solution or test sample. In accordance with this aspect, the method comprises the steps as set forth in the second aspect and further comprises the step of: f) adjusting the flow speed of the fluid to control the duration of the PCR cycling. In one embodiment, the flow speed is monitored and adjusted

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such that the desired number of PCR cycles is completed while the test solution is moving through the defined section of the channel. That is, the flow speed is adjusted to determine the duration of the PCR cycling. In another embodiment, this monitoring and adjustment is performed by an appropriately programmed computer. In a further embodiment, the flow rate is adjusted by regulating the pressure at the input end of the channel. In an additional embodiment, the flow rate is adjusted by regulating the pressure regulated vacuum at the output end of the channel. In another embodiment, the flow rate is adjusted by regulating a pump, such as a syringe pump.

[0021] In a fourth aspect, the present invention provides method for monitoring the progress of a polymerase chain reaction in a channel comprising the steps of: a) moving a bolus of test solution containing real-time PCR reagents in a channel; b) moving a carrier fluid in said channel, sequentially alternating with a test bolus; c) cycling the temperature in a defined section of the channel in order to achieve PCR; d) capturing an image of a reaction-dependent fluorescence signal along a section of the channel; e) measuring the average flow speed in the channel; and f) relating position of the test bolus to the number of temperature cycles experienced by a test bolus from the average flow rate. In one embodiment, the average flow speed is measured by comparing sequential images of the reaction-dependent fluorescent signal from the channel. In a second embodiment, the average flow speed is measured by comparing sequential images of a reaction-independent flow marker from the channel. The reaction-independent flow marker may be (i) pre-mixed in the test bolus, (ii) introduced to the channel as a bolus alternating with the test bolus or (iii) pre-mixed in the carrier fluid. In another embodiment, scattered light from the reaction-independent flow marker is resolvable from the reaction-dependent fluorescence by wavelength spectrum. In an alternative embodiment,

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scattered light from the reaction-independent flow marker is resolvable from the reaction-dependent fluorescence on the basis of fluorescence lifetime. In another embodiment, the reaction-independent flow marker is further used to determine the flow dispersion of the test bolus. In a further embodiment, the image of reaction-dependent fluorescence is captured at least once per PCR cycle. In one embodiment, the image of reaction-dependent fluorescence is captured sequentially by scanning a length of the channel on a time scale shorter than the duration of one PCR cycle. In an alternative embodiment, the image of reaction-dependent fluorescence is captured by acquiring signal from multiple points along the channel simultaneously. In another embodiment, the flow speed measurements are part of a feedback loop for regulating the flow speed. In a further embodiment, the flow speed is measured through detecting a sample bolus entrance into and exit from a defined section of the channel.

[0022] In a fifth aspect, the present invention provides a method for monitoring the progress of a polymerase chain reaction in a channel, comprising the steps of: a) moving a bolus of test solution containing real-time PCR reagents in a channel; b) moving a carrier fluid in the same channel, sequentially alternating with a test bolus; c) applying spatial temperature zones along the channel to achieve PCR; d) monitoring the fluorescence signal at fixed spatial locations along the channel corresponding to fixed points in the PCR cycle; e) measuring the average flow speed of the fluid in the channel; and f) adjusting the flow rate in order to control the timing of the PCR cycle. In one embodiment, the temperature is cycled using a thermal transfer element along portions of the channel. In another embodiment, the thermal transfer element cycles the temperature in the portions of the channel. In a further embodiment, an appropriately programmed computer controls the temperature cycling of the thermal transfer element. In one embodiment, the temperature of the thermal transfer element is detected and fed back to the

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computer. In accordance with these embodiments, heating and cooling are applied to portions of the channel such that a PCR temperature profile is followed. The test boluses are pumped through this reaction zone at a flow rate (speed) such that the number of PCR cycles required is achieved during the time the bolus flows from the upstream end to the downstream end of the reaction zone. By monitoring the flow speed in the channel in this aspect of the invention, the duration of the PCR cycle period can be determined.

[0023] In a sixth aspect, the present invention provides systems and/or kits adapted for practicing the methods described herein. The systems and/or kits can include system instructions (e.g., embodied in a computer or in a computer readable medium, e.g., as system software) for practicing any of the method steps herein. Fluid handling elements for storing, transferring, aliquotting, or diluting samples, e.g., microfluidic handling elements, and detector elements can also be components of the systems and kits herein. In addition, packaging materials, integration elements (e.g., instrument cases, power supplies, etc.), instructions for using the systems and kits and the like can be features of the invention.

[0024] In one embodiment, the invention provides a system that includes a microfluidic device comprising one or more amplification channels each configured to thermocycle one or more test solutions. A thermal transfer element for cycling the temperature along a defined section of the channel(s) is also included. The thermal transfer element is integral with or proximal to the microfluidic device. A source of illumination integral with or proximal to the microfluidic device is also included, where the source of illumination is configured to illuminate the channel(s). A detector integral with or proximal to the microfluidic device is also included, where the detector is configured to detect the amplification products and/or marker in the channel(s). In one embodiment, the detector can independently detect signals from two or more

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detectable markers with different signals; e.g., a detector that can simultaneously detect fluorescent or other emissions at two or more wavelength ranges. The system includes one or more elements for controlling the flow rate of the continuously moving test solution and carrier fluid in the channel(s), as well as system instruction or software for monitoring and controlling the flow rate in order to control the timing of each PCR cycle. Typically, the system includes a sensor to detect the temperature of the thermal transfer element and to provide feedback information for controlling the temperature cycles. Typically, the system also includes system software for generating and processing data, such as timing the detection of the amplified products and/or marker, monitoring location of test solutions in the channel(s) and the like.

[0025] In a second embodiment, the system may also include a dilution module that dilutes the sample into multiple test solutions, as well as system instructions that direct the dilution module to aliquot the sample into a plurality of test solutions. The dilution module may be integral with or proximal to the microfluidic device.

[0026] The system optionally comprises software with instructions for performing any of the method steps described herein. For example, the system can include statistical or probabilistic system software that performs one or more statistical or probabilistic analysis of signals received from one or more of the test solutions subjected to thermocycling. For example, the statistical or probabilistic analysis can include Poisson analysis, Monte Carlo analysis, application of a genetic algorithm, neural network training, Markov modeling, hidden Markov modeling, multidimensional scaling, PLS analysis, and/or PCA analysis. The statistical or probabilistic analysis optionally comprises quantitatively determining a concentration, proportion, or number of the nucleic acids of interest in the sample.

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[0027] The system also optionally includes fluid handling or storage features such as sample storage modules that store the samples until they are to be diluted by the dilution module, a sample retrieval module that retrieves the sample from the sample storage module and delivers it to the dilution module, or the like. These features are optionally designed to provide for continuous flow of fluid (e.g., comprising the sample) through the system (thereby providing for higher sample throughput). The system may also include means and system software for analyzing the amplified product. In one embodiment, the system may include system software that correlates a reproducible signal shape, length, width, volume or area occupied by the amplification products, as detected by the detector, to (a) the number of copies of the nucleic acid of interest present in one of the test solutions, or to the number of copies of the nucleic acid of interest present in the sample, or both or (b) an identification of the amplification product(s) present in the sample or (c) any other analytical parameters known and used in the art.

[0028] Many of the above methods or systems can be used in combination. Additional features of the invention will become apparent upon review of the following description.

BRIEF DESCRIPTION OF THE FIGURES

[0029] The file of this patent contains at least one drawing executed in color photograph. Copies of this patent with color photograph(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

[0030] Figure 1 shows a block diagram of a real-time PCR architecture in accordance with one embodiment of the present invention.

[0031] Figure 2 shows a schematic illustration of a real-time PCR microfluidic system in accordance with one embodiment of the present invention.

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[0032] Figures 3A and 3B shows a sensor output image with data processing from the system of Figure 2.

[0033] Figure 4 shows a schematic illustration of a real-time PCR microfluidic system in accordance with another embodiment of the present invention.

[0034] Figure 5 shows sensor output data from the system of Figure 4 in accordance with the present invention.

[0035] Figure 6 shows a system that is useful for quantitative PCR in real-time in a microfluidic device in accordance with yet another embodiment of the present invention.

[0036] Figure 7 shows a process diagram illustrating a mode of operation according to one embodiment of the invention.

[0037] Figure 8A is an image of a fluorescent signal showing the growth of PCR products in microfluidic channels under flow conditions.

[0038] Figure 8B is a plot of the intensity of the marker along a microchannel from Figure 8A.

[0039] Figure 9A is an image of a fluorescent signal showing the growth of PCR products in microfluidic channels under flow conditions.

[0040] Figure 9B is a plot of the intensity of the green signal along a microchannel from Figure 9A.

DETAILED DESCRIPTION OF THE INVENTION

[0041] The present invention relates to methods for amplifying nucleic acids in micro-channels. More specifically, the present invention relates to methods for performing a real-time

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polymerase chain reaction (PCR) in a continuous-flow microfluidic system and to methods for monitoring real-time PCR in such systems.

[0042] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, the preferred materials and methods are described herein.

[0043] Figure 1 illustrates a block diagram of a real-time PCR architecture in accordance with one embodiment of the present invention. The system includes a test solution reservoir 101, which as described above, may be a reservoir containing multiple test solutions, such as a microtiter plate in which each well contains different test solutions, e.g., test samples. The system further includes a carrier fluid reservoir 102. In one embodiment, the test solution is substantially the same as the carrier fluid, except that the test solution comprises all the necessary real-time PCR reagents. The real-time PCR reagent mixture may include PCR primers, dNTPs, polymerase enzymes, salts, buffers, surface-passivating agents, and the like. In addition, the real-time PCR mixture may include a non-specific fluorescent DNA detecting molecule, a sequence-specific fluorescent DNA probe or a marker. In an additional embodiment, the carrier fluid is an immiscible fluid (such as an oil, a fluorinated liquid, or any other nonaqueous or hydrophobic solvent). The purpose of the carrier fluid is to deter transfer of material from one test bolus to another. Another purpose of the carrier fluid is to provide a distinguishable transition between boluses that may be used to track the fluid flow in the channel. In one embodiment, the carrier fluid may include a marker.

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[0044] In one embodiment, the test solution and carrier fluid are introduced into a microchannel 103 through a switch 104. The dimensions of the microchannel are small enough to allow for the amplification and detection of a single DNA molecule originally present in the test solution. Microchannel 103 may be a single microchannel or it may be one of several microchannels that are part of a microfluidic device (such as shown in Figures 2, 4 and 6). Switch 104 is under control of a main control and processing computer 105 such that the carrier fluid and the test solution are sequentially alternately introduced into microchannel 103. The volume of the test solution and carrier fluid that is introduced into microchannel 103 is selected such that there is minimal blending between them during movement through microchannel 103.

[0045] A multitude of reactions in series (or sequential reactions) can thus be carried out in the same microchannel 103 as a result of the continuous movement of different test solutions through microchannel 103 each separated by the carrier fluid. If microchannel 103 is one of several microchannels in a microfluidic device, then a multitude of reactions can also be carried out in parallel in the microchannels of the microfluidic device. The flow rate of the carrier fluid and test solution through microchannel 103 is controlled by pump mechanism 106. Pump mechanism 106 is under control of main control and processing computer 105 in order to regulate the flow rate of the test solution and the carrier fluid in microchannel 103. The flow rate is regulated such that a desired number of PCR cycles are performed as the test solution passes through microchannel 103.

[0046] Pump mechanism 106 can regulate the flow rate of the test solution and carrier fluid by positive pressure at the upstream side or inlet of microchannel 103 or by negative pressure at the downstream side or outlet of microchannel 103. In one embodiment, the pressure difference is approximately 1 psi, although other pressure differences may be utilized. The length of

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microchannel 103 is suitable for the completion of the desired number of PCR cycles such as, for example, 10 to 50 cycles of PCR, or any number in between, as the test solution moves through the reaction zone of microchannel 103. The reaction zone of microchannel 103 is the region of the microchannel in which the temperature is cycled for conducting the PCR cycles. Typically, 25-30, 25-35, 25-40, 30-35, 30-40 or 35-40 PCR cycles are performed for standard amplification reactions. The length of microchannel 103 to accomplish the desired number of PCR cycles is also dependent on the volume of test solution and carrier fluid that are sequentially alternately moved through microchannel 103. For example, if the reaction zone is 40 mm, then the minimum volume of test solution would occupy 1 mm in the reaction zone of microchannel 103, and the maximum volume of test solution would occupy 20 mm in the reaction zone of microchannel 103. Thus, in this non-limiting example, a minimum of 1 sample and a maximum of 20 samples could be amplified as the test solution moves through the reaction zone of microchannel 103. Of course, the microchannel length and sample volumes can be selected to amplify any number of samples.

[0047] A temperature control system 107 is included in the system to control and cycle the temperature to produce suitable temperatures for the PCR cycles as the test solution moves through microchannel 103. Suitable temperatures for the PCR cycles are well known to skilled artisan and may include a first temperature in the range of about 85° C to about 100° C, a second temperature in the range of about 20° C to about 70° C and a third temperature in the range of about 55° C to about 80° C. Temperature control system 107 is integral with or proximal to microchannel 103 or to the microchannels of a microfluidic device. Temperature control system 107 includes heaters 108, coolers 109, temperature sensors 110 and a temperature controller 111. Temperature controller 111 collects temperature information from the temperature sensors 110

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and generates control signals based on the temperature information. The control signals are sent to the heaters 108 or coolers 109 to cycle the temperature in microchannel 103 for the desired PCR cycles. Temperature controller 111 is under control of main control and processing computer 105 so that the temperature is cycled such that the desired number of PCR cycles is performed as the test solution moves through microchannel 103. The different PCR reactions in the different test solutions follow one after another in microchannel 103 in a serial manner due to the continuous flow in microchannel 103.

[0048] In one embodiment, the heating and cooling may be accomplished by blowing air of the appropriate temperatures. In another embodiment, the heating and cooling may be accomplished by circulating water or fluid baths. In an additional embodiment, the heating and cooling may be accomplished by Peltier-effect elements which are well known to the skilled artisan. Junctions of different metals, which are crossed by an electric current, make it possible to cool or heat a small surface. A temperature probe on the Peltier element makes it possible to regulate the power, which is proportional to the electrical intensity, and thus makes it possible to regulate the temperature. In a further embodiment, a metal block is used as a thermal transfer element and includes a thermoelectric cooler in contact with the metal block. This metal block can be made of any metal (or metal alloy) having suitable thermal transfer properties such as, for example, aluminum. In order to reduce backscatter of light from the metal block it is preferably painted black or anodized. The temperatures for the PCR cycles can equilibrate at suitable time intervals for this thermal transfer unit. For example, the temperatures for the PCR cycles can equilibrate within approximately 1-2 seconds or even faster.

[0049] In one embodiment, temperature control system 107 cycles the temperature in an entire defined section of microchannel 103, i.e., that section of microchannel 103 in which the

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PCR cycles are performed. This defined section of microchannel 103 is also known as the reaction zone. Thus, in this embodiment a constant temperature zone is used to provide the thermal cycling. An appropriately programmed computer controls the temperature cycling of the thermal transfer element. In accordance with this embodiment, heating and cooling are applied to a length of the channel (the reaction zone) such that its temperature follows a PCR profile in time. The test boluses are pumped through this reaction zone at a flow rate (speed) such that the number of PCR cycles required is achieved during the time the bolus flows from the upstream end to the downstream end of the reaction zone.

[0050] In a second embodiment, temperature control system 107 applies spatial temperature zones along microchannel 103 to achieve a polymerase chain reaction. In one aspect, the temperature is cycled using a thermal transfer element along portions of the microchannel 103 or the microchannels of a microfluidic device. In another aspect, the thermal transfer element cycles the temperature in portions of microchannel 103. An appropriately programmed computer controls the temperature cycling of the thermal transfer element. In accordance with this embodiment, heating and cooling are applied to portions of the channel such that a PCR temperature profile is followed. The test boluses are pumped through this reaction zone at a flow rate (speed) such that the number of PCR cycles required is achieved during the time the bolus flows from the upstream end to the downstream end of the reaction zone.

[0051] An optical imaging system 112 is included in one embodiment of the present invention to detect the amplification product and to monitor the flow rate of the test solution in microchannel 103. In one embodiment, the optical imaging system 112 is a fluorescent imaging system that preferably includes one or more excitation sources 113, one or more optics/filters 114 and one or more detectors 115. In one embodiment, excitation sources 113 generate light at

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desired wavelengths to excite the labels used for detecting the amplification products during real-time PCR and/or to detect markers that may be present to monitor the flow rate of the test solution in microchannel 103. Optics/filters 114 are used to form a beam of light and/or to direct the light from excitation sources 113 to the appropriate positions on the microchannel 103. Optics/filters 114 are also used to filter the light to exclude light of undesired wavelengths or to reduce backscatter from reaching detectors 115. The desired wavelengths to excite the labels used in real-time PCR will depend on the precise labels and/or markers used, e.g., intercalating dyes, molecular beacons, quantum dots or TaqMan® probes, which wavelengths are well known to skilled artisans. Similarly, the emission wavelengths of the precise labels and/or markers are well known to skilled artisans. Detectors 115 detect the emission wavelengths of the excited labels and/or markers and measure the intensity of the emitted light. Optical imaging system 112 preferably is able to distinguish between multiple microchannels in a microfluidic device.

[0052] Optical imaging system 112 is under control of main control and processing computer 105 which directs the optical/fluorescence imaging system 112 to measure the intensity of the emitted light at desired time intervals, such as, for example, at least once during each PCR cycle at a plurality of locations in microchannel 103 or in the microchannels of a microfluidic device. Detectors 115 generate a signal or an image of the intensity of the emitted light and direct it to main control and processing computer 105 for analysis of the amplification product and for monitoring the flow rate of the test solution. Detectors 115 may include multiple-pixel array detectors (such as a CCD detector) and/or discrete single-pixel or non-imaging detectors. Detectors 115 may be integral with or proximal to microchannel 103 or to the microchannels of a microfluidic device. Detectors 115 may be stationary or may be scanning. The detectors 115 should have appropriate resolution for obtaining meaningful results and for monitoring of fluid

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flow in microchannel 103, particularly because the fluid is continuously moving in microchannel 103.

[0053] As described above, the real-time PCR mixture may include a non-specific fluorescent DNA detecting molecule (such as an intercalating dye), a sequence-specific fluorescent DNA probe (such as a molecular beacon, a TaqMan® probe or a quantum dot probe), or a flow marker (such as a quantum dot), and the carrier fluid may include a flow marker. In one embodiment, the optical imaging system 112 is utilized to detect the intensity of the fluorescence from the DNA detecting molecule or the probe (i.e., the intensity of the fluorescent signal) and/or to detect the fluorescence of the marker. The fluorescence of the marker can be used to delineate the test solution from the carrier fluid and can also be used to determine and monitor the flow speed of the test solution or carrier fluid. The intensity of the fluorescent signal can be used to detect amplified product, to determine the quantity of amplified product, to determine the number of original molecules present in the test solution, and the like as well known to a skilled artisan for real-time PCR. The intensity of the fluorescent signal can also be used to determine and monitor the flow speed of the test solution.

[0054] In one embodiment, the intensity of the fluorescent signal is measured (e.g., an image of the fluorescent signal is taken) at a specific time and/or temperature during the PCR temperature cycle. In another embodiment, the intensity of the fluorescent signal is measured once during each PCR cycle. The optimal time to capture the image depends on the chemistry utilized. For example, if an intercalating dye is used to detect amplified product, the image should be captured at the end of the extension phase of the PCR cycle. If a TaqMan® probe is used to detect the amplified product, the image could be captured at any time during the PCR cycle. Main control and processing computer 105 can be programmed to take the image at the

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time and temperature desired. In a further embodiment, the intensity of the fluorescent signal is measured at a plurality of locations. The plurality of locations at which the intensity of the fluorescent signal is measured may be different sections of the microchannel. The plurality of locations at which the intensity of the fluorescent signal is measure may be the entire defined section (i.e., reaction zone) of the microchannel. In another embodiment, an image of at least one fluorescent signal along the length of the channel is made. In a further embodiment, the image capture is performed repeatedly on consecutive temperature cycling periods. The image may be created or the intensity of the fluorescent signal measured using a multiple-pixel array detector (such as a CCD or CMOS image sensor) or a single pixel detector. Fluorescent light may be collected using large field-of-view imaging optics and/or small field-of-view optics such as a fiber-optic/lens combination. A stationary mechanism or a scanning mechanism or both may be used to capture the image. The data concerning the intensity of the fluorescent signal is processed by an appropriately programmed computer.

[0055] After test solution has moved through microchannel 103 and completed the desired number of PCR cycles, it may optionally be sent to a post-PCR analyzer 116. Post-PCR analyzer 116 may include any analytical technique that can be used on PCR amplification products. Such techniques include, but are not limited to, sequencing, electrophoresis, probing, melt curve analysis, and the like.

[0056] Examples of systems useful for real-time PCR in microchannels in accordance with the present invention are shown schematically in Figures 2, 4 and 6. In Figure 2, a real-time PCR system 200 is illustrated with a microfluidic device 201 that contains multiple microchannels. Because there is an effect of gravity on the fluid in the microchannels, microfluidic device 201 is preferably oriented such that the test and carrier fluids flow in a

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generally horizontal direction. However, microfluidic device 201 may be oriented in such that the carrier and test fluids flow in other directions including the vertical direction. A thermal transfer element is located proximal to microfluidic device 201. The optical imaging system includes an illumination laser 202 and a beam forming lens 203 for illuminating the microchannels in microfluidic device 201 with the appropriate excitation wavelength(s). The optical imaging system further includes a filter 204 for filtering the emission wavelength(s) and reducing backscatter and a multiple-pixel array detector 205. The real-time PCR system 200 may also optionally include a lens 207. A sensor output image with data processing using this system is shown in Figures 3A and 3B. Figure 3A shows a two dimensional image of 8 parallel microfluidic channels filled with 100 nM FAM fluorescent dye. Figure 3B shows a profile plot of the region of interest box 301 of Figure 3A. .

[0057] In Figure 4, a system 400 for real-time PCR is illustrated with a microfluidic device 401 that contains multiple microchannels. A thermal transfer element (not shown) is proximal to microfluidic device 401. The optical imaging system includes an illumination laser 402 and a beam forming lens 404 for illuminating the microchannels in microfluidic device 401 with the appropriate excitation wavelength(s). The optical imaging system further includes a filter 405 for filtering the emission wavelength(s) and reducing backscatter and a CCD detector 406. The real-time PCR system 400 may also optionally include a lens 403. Sensor output data from a 512 pixel linear CCD array sensor using this system is shown in Figure 5. The microchannels were filled with 100 nM FAM fluorescent dye to demonstrate this sensor output data. The peaks show the fluorescence from the microchannel. In this embodiment, the laser beam and detector field of view are scanned with respect to the length of the microchannel, in order to measure fluorescence as a function of position along the channel

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[0058] A system useful for real-time PCR in a microfluidic device is illustrated in Figure 6 which is capable of multi-color sensing from multiple fluorophores. In this embodiment, real-time PCR system 600 includes a microfluidic device 602 which preferably includes multiple microchannels and a thermal element (not shown) located proximal to microfluidic device 602. This embodiment further includes a light source 608 that is capable of emitting light at multiple wavelengths such as, for example, red and blue light, for providing multi-color excitation light to the channels in microfluidic device 602. In one aspect of this embodiment, the light source 608 includes multiple LEDs which emit light at different wavelengths. The real-time PCR system 600 further includes an excitation filter 607 and a dual bandpass interference filter 605 that passes light at the multiple excitation wavelengths (such as red light and blue light). The system 600 also includes a lens 601 for collecting the emitted light, for example, from a flourophore in the amplification product and a different fluorophore in a flow marker, and directing it to detector 604. In one embodiment, an emission filter 606 is also provided for blocking excitation light and passing the fluorescent light from the flourophores. Detector 604 can be a multiple pixel array which is capable of multicolor sensing such as, for example, CMOS image sensor.

[0059] In a preferred embodiment, the system 600 further includes a color filter array 610 for arranging RGB colors on the detector 604 which enables simultaneous imaging of multiple colors. The color filter array 610 could be, for example, a Bayer filter. The multi-color system of the present invention enables simultaneous imaging of multiple colors which provides, for example, DNA and flow marker detection utilizing different fluorophores.

[0060] The systems described herein are specifically adapted for methods of performing real-time PCR in a microchannel and methods for monitoring the progress of a polymerase chain reaction in a microchannel. In one embodiment of the methods in accordance with the present

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invention, a bolus of test solution is continuously moved in the microchannel. The test solution contains real-time PCR reagents as described above. A carrier fluid is continuously moved in the microchannel. The carrier fluid sequentially alternates with a test bolus. The temperature is cycled in a defined section of the microchannel in order to achieve PCR. The intensity of the fluorescent signal is measured at a plurality of locations along the defined section of the microchannel. Thus, in accordance with this embodiment, a method for performing real-time PCR comprises the steps of: a) continuously moving a bolus of test solution containing real-time PCR reagents in a channel; b) moving a carrier-fluid in the channel, sequentially alternating with a test bolus; c) cycling the temperature in a defined section of the channel in order to achieve PCR; and d) measuring the intensity of the fluorescent signal at a plurality of locations along the defined section of the channel.

[0061] In one embodiment, the temperature is cycled using a thermal transfer element along the defined section of the channel. In another embodiment, the thermal transfer element cycles the temperature in the entire defined section of the channel. That is, a constant temperature zone is used to provide the thermal cycling. In a further embodiment, an appropriately programmed computer controls the temperature cycling of the thermal transfer element. In one embodiment, the temperature of the thermal transfer element is detected and fed back to the computer. In accordance with these embodiments, heating and cooling are applied to a length of the channel (the reaction zone) such that its temperature follows a PCR profile in time. The test boluses are pumped through this reaction zone at a flow rate (speed) such that the number of PCR cycles required is achieved during the time the bolus flows from the upstream end to the downstream end of the reaction zone.

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[0062] In another embodiment, the intensity of the fluorescent signal is measured at a specific time and/or temperature during the PCR temperature cycle. In an additional embodiment, the intensity of the fluorescent signal is measured once during each PCR cycle. In a further embodiment, the plurality of locations at which the intensity of the fluorescent signal is measured is the entire defined section of the channel. In another embodiment, the data concerning the intensity of the fluorescent signal is processed by an appropriately programmed computer. In another embodiment, an image of at least one fluorescent signal along the length of the channel is made. In a further embodiment, the image capture is performed repeatedly on consecutive temperature cycling periods. The image may be created or the intensity of the fluorescent signal measured using a multiple-pixel array detector. A stationary mechanism or a scanning mechanism or both may be used to capture the image.

[0063] In a second embodiment of the methods in accordance with the present invention, the performance of real-time PCR includes monitoring the flow rate (flow speed) of the fluid in the microchannel. The liquid may be the test solution or the carrier fluid. By monitoring the flow rate in the channel, the location of a test bolus as a function of time can be determined, thus identifying the number of PCR cycles that has been experienced by the test bolus. Thus, in accordance with this embodiment, a method for performing real-time PCR comprises the steps of: a) continuously moving a bolus of test solution containing real-time PCR reagents in a channel; b) moving a carrier-fluid in the channel, sequentially alternating with a test bolus; c) cycling the temperature in a defined section of the channel in order to achieve PCR; d) measuring the intensity of the fluorescent signal at a plurality of locations along the defined section of the channel and e) measuring the average flow rate of the fluid in the channel.

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[0064] In one embodiment, average flow speed of the fluid is measured by comparing sequential images of the reaction-dependent fluorescent signal from the channel. In a second embodiment, the average flow speed of the fluid is measured by comparing sequential images of a marker (i.e., a reaction-independent marker) in the channel. The use of the marker has the advantage that if the real-time PCR signal is not detectable, the fluid flow would still be detectable. Examples of potential markers are dyes, semiconductor quantum dots, polymer microbeads, scattering metal particles, microbubbles, and the like known to skilled artisans. In one embodiment, the marker is present in the test solution. If the marker is present in the test solution, it should not adversely affect any PCR chemical reactions. In another embodiment, the marker is present in the carrier fluid. In a further embodiment, the marker is a separate bolus in series with test boluses. In an additional embodiment, the marker is resolvable from the fluorescent signal, such as by excitation wavelength, emission spectrum, lifetime, and the like well known in the art. In a further embodiment, the data concerning the sequential images of the intensity of the fluorescent signal or of the marker is processed by an appropriately programmed computer. In accordance with these embodiments, the average flow speed is determined by comparing images taken from consecutive PCR cycles. In a still further embodiment, the flow rate can be measured using a reaction zone entrance detector and a flow speed meter. Flow speed could be estimated, for example, by knowing the dimensions of the channel and measuring the volume flow rate.

[0065] In a third embodiment of the methods in accordance with the present invention, the performance of real-time PCR includes monitoring the flow rate (flow speed) of the fluid in the microchannel and adjusting the flow rate as necessary to control the duration of the PCR cycling experienced by a test solution. Thus, in accordance with this embodiment, a method for

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performing real-time PCR comprises the steps of: a) continuously moving a bolus of test solution containing real-time PCR reagents in a channel; b) moving a carrier-fluid in the channel, sequentially alternating with a test bolus; c) cycling the temperature in a defined section of the channel in order to achieve PCR; d) measuring the intensity of the fluorescent signal at a plurality of locations along the defined section of the channel; e) measuring the average flow speed of the fluid in the channel; and f) adjusting the flow rate of the fluid to control the duration of the PCR cycling.

[0066] In one embodiment, the flow rate is monitored and adjusted such that the desired number of PCR cycles is completed while the test solution is moving through the defined section of the channel. That is, the flow rate is adjusted to determine the duration of the PCR cycling. In another embodiment, this monitoring and adjustment is performed by an appropriately programmed computer, such as main control and processing computer 105. In a further embodiment, the flow rate is adjusted by regulating the pressure at the input end of the channel. In an additional embodiment, the flow rate is adjusted by regulating the pressure regulated vacuum at the output end of the channel. In another embodiment, the flow rate is adjusted by regulating a pump, such as a syringe pump. In one embodiment, the flow rate is monitored and adjusted so that the desired number of PCR cycles is performed as the test solution moves through the reaction zone of the microchannel. For example, if the reaction zone of the microchannel is 30 mm and it is desired to perform 30 PCR cycles, then the flow rate is monitored so that a flow rate of 1 mm/cycle is achieved and maintained. Since each PCR cycle may be on the order of 30-60 seconds, the flow speed in the microchannel is fairly slow in this example.

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[0067] Figure 7 is a process diagram illustrating a mode of operation for regulating the flow speed of the fluid as moves through the channels according to one embodiment of the invention. In this embodiment, a closed loop feedback is used to regulate the flow speed. First, the fluid in the channels is moved by activating a regulated pressure pump, such as, for example, pump mechanism 106, at a pump pressure set point. As the fluid moves through the channel, a PCR temperature profile is executed over a defined section of a channel. At an appropriate time during the PCR temperature cycle, the system acquires one or more images of the contents of the channel. These image data and the time of acquisition are stored to a database for subsequent analysis, such as, for example, the main control and processing computer 105. If two sequential images are not available, the temperature cycling and image acquisition repeat without changing the pressure set point. If two sequential images are available, they may be compared to determine how far the fluid has moved along the channel. Dividing the average displacement by the time elapsed gives an average flow speed. If there is a difference between the measured flow speed and the desired flow speed, the pump pressure set point is adjusted automatically so that the desired flow speed obtained, and the process is repeated. If there is no difference between the measured flow speed and the desired flow speed, the pump pressure set point remains the same, and the process is repeated.

[0068] The above described systems are specifically adapted for methods for monitoring the progress of a polymerase chain reaction in a microchannel. In accordance with one embodiment, a method for monitoring the progress of a polymerase chain reaction in a microchannel comprises the steps of: a) moving a bolus of test solution containing real-time PCR reagents in a microchannel; b) moving a carrier fluid in said microchannel, sequentially alternating with a test bolus; c) cycling the temperature in a defined section of the microchannel in order to achieve

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PCR; d) capturing an image of a reaction-dependent fluorescence signal along a section of the microchannel; e) measuring the average flow rate in the microchannel; and f) relating position of the test bolus to the number of temperature cycles experienced by a test bolus from the average flow rate.

[0069] In one embodiment, the average flow rate is measured by comparing sequential images of the reaction-dependent fluorescent signal from the channel. In a second embodiment, the average flow rate is measured by comparing sequential images of a reaction-independent flow marker from the channel. The reaction-independent flow marker may be (i) pre-mixed in the test bolus, (ii) introduced to the channel as a bolus alternating with the test bolus or (iii) pre-mixed in the carrier fluid. In another embodiment, scattered light from the reaction-independent flow marker is resolvable from the reaction-dependent fluorescence by wavelength spectrum. In an alternative embodiment, scattered light from the reaction-independent flow marker is resolvable from the reaction-dependent fluorescence on the basis of fluorescence lifetime. In another embodiment, the reaction-independent flow marker is further used to determine the flow dispersion of the test bolus. In a further embodiment, the image of reaction-dependent fluorescence is captured at least once per PCR cycle. In one embodiment, the image of reaction-dependent fluorescence is captured sequentially by scanning a length of the channel on a time scale shorter than the duration of one PCR cycle. In an alternative embodiment, the image of reaction-dependent fluorescence is captured by acquiring signal from multiple points along the channel simultaneously. In another embodiment, the flow rate measurements are part of a feedback loop for regulating the flow rate. In a further embodiment, the flow rate is measured through detecting a sample bolus entrance into and exit from a defined section of the channel.

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[0070] In accordance with another embodiment, a method for monitoring the progress of a polymerase chain reaction in a microchannel comprises the steps of: a) moving a bolus of test solution containing real-time PCR reagents in a microchannel; b) moving a carrier fluid in the same microchannel, sequentially alternating with a test bolus; c) applying spatial temperature zones along the microchannel to achieve PCR; d) monitoring the fluorescence signal at fixed spatial locations along the microchannel corresponding to fixed points in the PCR cycle; e) measuring the average flow rate of the fluid in the microchannel; and f) adjusting the flow rate in order to control the timing of the PCR cycle.

[0071] In one embodiment, the temperature is cycled using a thermal transfer element along portions of the channel. In another embodiment, the thermal transfer element cycles the temperature in the portions of the channel. In a further embodiment, an appropriately programmed computer controls the temperature cycling of the thermal transfer element, such as main control and processing computer illustrated in Figure 1. In one embodiment, the temperature of the thermal transfer element is detected and fed back to the computer. In accordance with these embodiments, heating and cooling are applied to portions of the channel such that a PCR temperature profile is followed. The test boluses are pumped through this reaction zone at a flow rate (speed) such that the number of PCR cycles required is achieved during the time the bolus flows from the upstream end to the downstream end of the reaction zone. By monitoring the flow rate in the channel in this aspect of the invention, the duration of the PCR cycle period can be determined.

[0072] In one embodiment, the present invention also provides systems and/or kits adapted for practicing the above described methods. As illustrated above, the systems and/or kits can include system instructions (e.g., embodied in a computer or in a computer readable medium,

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e.g., as system software) for practicing any of the method steps herein. Fluid handling elements for storing, transferring, aliquotting, or diluting samples, e.g., microfluidic handling elements, and detector elements can also be components of the systems and kits herein. In addition, packaging materials, integration elements (e.g., instrument cases, power supplies, etc.), instructions for using the systems and kits and the like can be features of the invention.

[0073] In one embodiment, the invention provides a system that includes a microfluidic device comprising one or more amplification channels (microchannels) each configured to thermocycle one or more test solutions. A thermal transfer element for cycling the temperature along a defined section of the channel(s) is also included. The thermal transfer element is integral with or proximal to the microfluidic device. A source of illumination integral with or proximal to the microfluidic device is also included, where the source of illumination is configured to illuminate the channel(s). A detector integral with or proximal to the microfluidic device is also included, where the detector is configured to detect the amplification products and/or marker in the channel(s). In one embodiment, the detector can independently detect signals from two or more detectable markers with different signals; e.g., a detector that can simultaneously detect fluorescent or other emissions at two or more frequencies, such as the system illustrated in Figure 6. The system includes one or more elements for controlling the flow rate of the continuously moving test solution and carrier fluid in the channel(s), as well as system instruction or software for monitoring and controlling the flow rate in order to control the timing of each PCR cycle. Typically, the system includes a sensor to detect the temperature of the thermal transfer element and to provide feedback information for controlling the temperature cycles. Typically, the system also includes system software for generating and processing data,

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such as timing the detection of the amplified products and/or marker, monitoring location of test solutions in the channel(s) and the like.

[0074] In one embodiment, the system may also include a dilution module that dilutes the sample into multiple test solutions, as well as system instructions that direct the dilution module to aliquot the sample into a plurality of test solutions. The dilution module may be integral with or proximal to the microfluidic device.

[0075] In one embodiment, the system optionally comprises software with instructions for performing any of the method steps described herein. For example, the system can include statistical or probabilistic system software that performs one or more statistical or probabilistic analysis of signals received from one or more of the test solutions subjected to thermocycling. For example, the statistical or probabilistic analysis can include Poisson analysis, Monte Carlo analysis, application of a genetic algorithm, neural network training, Markov modeling, hidden Markov modeling, multidimensional scaling, partial least square (PLS) analysis, and/or principle component analysis (PCA). The statistical or probabilistic analysis optionally comprises quantitatively determining a concentration, proportion, or number of the nucleic acids of interest in the sample. These statistical evaluations can be used to correlate abundance or proportions to diagnosis or prognosis associated with the diagnosis or prognosis.

[0076] In one embodiment, the system also optionally includes fluid handling or storage features such as sample storage modules that store the samples until they are to be diluted by the dilution module, a sample retrieval module that retrieves the sample from the sample storage module and delivers it to the dilution module, or the like. These features are optionally designed to provide for continuous flow of fluid (e.g., comprising the sample) through the system (thereby providing for higher sample throughput). The system may also include means and system

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software for analyzing the amplified product. In one embodiment, the system may include system software that correlates a reproducible signal shape, length, width, volume or area occupied by the amplification products, as detected by the detector, to (a) the number of copies of the nucleic acid of interest present in one of the test solutions, or to the number of copies of the nucleic acid of interest present in the sample, or both or (b) an identification of the amplification product(s) present in the sample or (c) any other analytical parameters known and used in the art.

[0077] The nucleic acid of interest that can be amplified and detected in the methods of the invention can be essentially any nucleic acid. The sequences for many nucleic acids and amino acids (from which nucleic acid sequences can be derived via reverse translation) are available. No attempt is made to identify the hundreds of thousands of known nucleic acids, any of which can be detected in the methods of the invention. Common sequence repositories for known nucleic acids include GenBank EMBL, DDBJ and the NCBI. Other repositories can easily be identified by searching the internet. The nucleic acid can be an RNA (e.g., where amplification includes RT-PCR) or DNA (e.g., where amplification includes PCR), or an analogue thereof (e.g., for detection of synthetic nucleic acids or analogues thereof). Any variation in a nucleic acid can be detected, e.g., a mutation, a single nucleotide polymorphism (SNP), an allele, an isotype, a fragment, a full-length nucleic acid, an amplicon, etc. Further, because the present invention is quantitative, one can detect variations in expression levels, fragmentation, or gene copy numbers by the methods.

[0078] In general, the methods of the invention are particularly useful in screening samples derived from patients for the nucleic acids of interest, e.g., from bodily fluids and/or waste from the patient. This is because samples derived from relatively large volumes of such materials can

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be screened in the methods of the invention (removal of such materials is also relatively non-invasive). The nucleic acids of interest (e.g., present in cancer cells) can easily comprise 1% or less of the related nucleic acid population of the sample (e.g., about 1%, 0.1%, 0.001%, 0.0001% or less of the alleles for a gene of interest). Thus, whole blood, serum, plasma, stool, urine, vaginal secretions, ejaculatory fluid, synovial fluid, a biopsy, cerebrospinal fluid, and amniotic fluid, sputum, saliva, lymph, tears, sweat, or urine, or the like, can easily be screened for rare nucleic acids or fragmentation by the methods of the invention, as can essentially any tissue of interest. These samples are typically taken, following informed consent, from a patient by standard medical laboratory methods.

[0079] Prior to amplification, nucleic acids are optionally purified from the samples by any available method, e.g., those taught in Berger and Kimmel (*Methods in Enzymology* **152**, Academic Press, Inc., San Diego, Calif., 1987); Sambrook and Russell, *Molecular Cloning*, 3rd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001); Ausubel et al., *Current Protocols in Molecular Biology* (John Wiley & Sons, updated through 2005). A plethora of kits are also commercially available for the purification of nucleic acids from cells or other samples (see, e.g., EasyPrep™, FlexiPrep™, both from Pharmacia Biotech; StrataClean™ from Stratagene; and, QIAprep™ from Qiagen). Alternately, samples can simply be directly subjected to amplification, e.g., following aliquotting and dilution. One advantage of single molecule detection is that the low concentration of sample components in the reaction can reduce the need for nucleic acid purification. That is, dilution of the sample reduces the abundance of unwanted components at the same time it distributes the nucleic acid of interest into reaction mixtures.

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[0080] One class of nucleic acids of interest to be detected in the methods herein is those involved in cancer. Any nucleic acid that is associated with cancer can be detected in the methods of the invention, e.g., those that encode over expressed or mutated polypeptide growth factors (e.g., sis), over expressed or mutated growth factor receptors (e.g., erb-B1), over expressed or mutated signal transduction proteins such as G-proteins (e.g., Ras), or non-receptor tyrosine kinases (e.g., abl), or over expressed or mutated regulatory proteins (e.g., myc, myb, jun, fos, etc.) and/or the like. In a preferred embodiment, specific or arbitrary nucleic acids of interest are screened for the amount of fragmentation, with high fragmentation generally associated with apoptosis of normal cells and less fragmentation associated, e.g., with sloughing of cancer cells. In general, cancer can often be linked to signal transduction molecules and corresponding oncogene products, e.g., nucleic acids encoding Mos, Ras, Raf, and Met; and transcriptional activators and suppressors, e.g., p53, Tat, Fos, Myc, Jun, Myb, Rel, and/or nuclear receptors. p53, colloquially referred to as the "molecular policeman" of the cell, is of particular relevance, as about 50% of all known cancers can be traced to one or more genetic lesion in p53.

[0081] One class of genes that are relevant to cancer is the class of nuclear hormone receptors, which have been described in detail and the mechanisms by which these receptors can be modified to confer oncogenic activity have been worked out. For example, the physiological and molecular basis of thyroid hormone action is reviewed in Yen (*Physiological Reviews* **81**:1097-1142, 2001), and the references cited therein. Known and well characterized nuclear receptors include those for glucocorticoids (GRs), androgens (ARs), mineralocorticoids (MRs), progestins (PRs), estrogens (ERs), thyroid hormones (TRs), vitamin D (VDRs), retinoids (RARs and RXRs), and the peroxisome proliferator activated receptors (PPARs) that bind eicosanoids. The so called "orphan nuclear receptors" are also part of the nuclear receptor superfamily, and

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are structurally homologous to classic nuclear receptors, such as steroid and thyroid receptors. Nucleic acids that encode any of these receptors, or oncogenic forms thereof, can be detected in the methods of the invention. About 40% of all pharmaceutical treatments currently available are agonists or antagonists of nuclear receptors and/or oncogenic forms thereof, underscoring the relative importance of these receptors (and their coding nucleic acids) as targets for analysis.

[0082] One class of nucleic acids of interest involved with cancer are those that are diagnostic of colon cancer, e.g., in samples derived from stool. Colon cancer is a common disease that can be sporadic or inherited. The molecular basis of various patterns of colon cancer is known in some detail. In general, germline mutations are the basis of inherited colon cancer syndromes, while an accumulation of somatic mutations is the basis of sporadic colon cancer. In Ashkenazi Jews, a mutation that was previously thought to be a polymorphism may cause familial colon cancer. Mutations of at least three different classes of genes have been described in colon cancer etiology: oncogenes, suppressor genes, and mismatch repair genes. One example nucleic acid encodes DCC (deleted in colon cancer), a cell adhesion molecule with homology to fibronectin. An additional form of colon cancer is an autosomal dominant gene, hMSH2 that comprises a lesion. Familial adenomatous polyposis is another form of colon cancer with a lesion in the MCC locus on chromosome #5. For additional details on Colon Cancer, see, Calvert et al. (*Annals of Internal Medicine* **137**:603-612, 2002) and the references cited therein. For a variety of colon cancers and colon cancer markers that can be detected in stool, see, e.g., Boland (*Reviews In Gastroenterological Disorders* **2** Supp. 1:S12-S19, 2002) and the references cited therein. As with other cancers, mutations in a variety of other genes that correlate with cancer, such as Ras and p53, are useful diagnostic indicators for cancer. In another aspect, detection of fragmentation levels using methods of the present invention can be particularly useful in

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detection of colon cancer. For example, as the amount of total patient DNA available in a stool specimen is low, the amplification aspect of the present invention can be beneficial to examination of the DNA. Whereas the DNA from cells sloughed from the normal colon lining is generally degraded into fragments, e.g., of about 100 base pairs in length, DNA entering the colon lumen from a colon tumor cells can remain generally unfragmented. Detecting the presence of a proportion of unfragmented nucleic acids over a certain threshold in a stool specimen can correlate to presence of a colon cancer.

[0083] Cervical cancer is another target for detection, e.g., in samples obtained from vaginal secretions. Cervical cancer can be caused by the human papilloma virus and has two oncogenes, E6 and E7. E6 binds to and removes p53 and E7 binds to and removes PRB. The loss of p53 and uncontrolled action of E2F/DP growth factors without the regulation of pRB is one mechanism that leads to cervical cancer. Furthermore, as with colon cancer, detecting the presence of a proportion of unfragmented nucleic acids over a certain threshold in a vaginal swab can correlate to the presence of a cervical cancer.

[0084] Other targets for detection by the methods of the invention include retinoblastoma, e.g., in samples derived from tears, and Neurofibromatosis Type 1, e.g. in CSF or via tissue sampling. Many other forms of cancer are known and can be found by detecting, e.g., associated genetic lesions, fragmentation proportions, or absolute concentrations of full-length nucleic acids of interest using the methods of the invention. Cancers that can be detected by detecting appropriate lesions or fragmentation values include cancers of the lymph, blood, stomach, gut, colon, testicles, pancreas, bladder, cervix, uterus, skin, and essentially all others for which an associated genetic lesion or fragmentation threshold exists. For a review of the topic, see, *The*

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Molecular Basis of Human Cancer, Coleman and Tsongalis (Eds), Humana Press, Totowa, New Jersey, 2001).

[0085] Similarly, nucleic acids from pathogenic or infectious organisms can be detected by the methods of the invention, e.g., for infectious fungi, e.g., *Aspergillus*, or *Candida* species; bacteria, particularly *E. coli*, which serves a model for pathogenic bacteria (and, of course certain strains of which are pathogenic), as well as medically important bacteria such as *Staphylococci* (e.g., *aureus*), or *Streptococci* (e.g., *pneumoniae*); protozoa such as sporozoa (e.g., *Plasmodia*), rhizopods (e.g., *Entamoeba*) and flagellates (*Trypanosoma, Leishmania, Trichomonas, Giardia*, etc.); viruses such as (+) RNA viruses (examples include Poxviruses e.g., vaccinia; Picornaviruses, e.g. polio; Togaviruses, e.g., rubella; Flaviviruses, e.g., HCV; and Coronaviruses), (-) RNA viruses (e.g., Rhabdoviruses, e.g., VSV; Paramyxovimses, e.g., RSV; Orthomyxovimses, e.g., influenza; Bunyaviruses; and Arenaviruses), dsDNA viruses (Reoviruses, for example), RNA to DNA viruses, i.e., Retroviruses, e.g., HIV and HTLV, and certain DNA to RNA viruses such as Hepatitis B. Single and low copy amplification methods of the invention can be useful in many cases, e.g., in exudates from bacterial infections to identify living (having full length nucleic acids) versus dead and lysed pathogens (having fragmented nucleic acids).

[0086] A variety of nucleic acid encoding enzymes (e.g., industrial enzymes) can also be detected according to the methods herein, such as amidases, amino acid racemases, acylases, dehalogenases, dioxygenases, diarylpropane peroxidases, epimerases, epoxide hydrolases, esterases, isomerases, kinases, glucose isomerases, glycosidases, glycosyl transferases, haloperoxidases, monooxygenases (e.g., p450s), lipases, lignin peroxidases, nitrile hydratases, nitrilases, proteases, phosphatases, subtilisins, transaminase, and nucleases. Similarly,

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agriculturally related proteins such as insect resistance proteins (e.g., the Cry proteins), starch and lipid production enzymes, plant and insect toxins, toxin-resistance proteins, Mycotoxin detoxification proteins, plant growth enzymes (e.g., ribulose 1,5-bisphosphate carboxylase/oxygenase), lipoxygenase (LOX), and phosphoenolpyruvate (PEP) carboxylase can also be detected.

[0087] The sample can be aliquotted and/or diluted using standard or microfluidic fluid handling approaches (or combinations thereof). Standard fluid handling approaches for dilution/aliquotting include, e.g., pipetting appropriate volumes of the sample into microtiter trays and adding an appropriate diluent. These operations can be performed manually or using available high throughput fluid handlers, such as, e.g., those designed to use serially dilute solutions in microtiter trays. High throughput equipment (e.g., incorporating automated pipettors and robotic microtiter tray handling) is preferred, as the present invention contemplates making and using high numbers of aliquots of a sample of interest.

[0088] Many automated systems for fluid handling are commercially available and can be used for aliquotting and/or diluting a sample in the context of the present invention. For example, a variety of automated systems are available from the Zymark Corporation (now Caliper Life Sciences, Hopkinton, MA), which typically include, e.g., robotics and fluid handling modules. Similarly, the common ORCA® robot, which is used in a variety of laboratory systems, e.g., for microtiter tray manipulation, is also commercially available, e.g., from Beckman Coulter, Inc. (Fullerton, Calif.). In any case, a conventional high throughput systems can be used in place of, or in conjunction with microfluidic systems (for example, conventional systems can be used to aliquot samples into microtiter trays, from which microfluidic systems can draw materials) in practicing the methods of the invention.

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[0089] Microfluidic systems provide a preferred fluid handling and amplification technology that can conveniently be applied to the present invention. In typical embodiments, such as those described above, samples are drawn into microfluidic devices that comprise networks of microscale cavities (channels, chambers, etc., having at least one dimension less than about 500 μM in size and often less than about 100 μM) and the samples are mixed, diluted, aliquotted or otherwise manipulated in the network of cavities (e.g., channels and/or chambers). For example, the microscale device can comprise one or more capillary, in fluid communication with the network, extending outward from a body structure of the microscale device. In one embodiment, negative pressure (vacuum) is applied to the capillary and fluids are drawn into the network from a container (e.g., a well on a microtiter tray). This process can be multiplexed by using a device that comprises multiple capillary channels, permitting many samples to be drawn into the network and processed simultaneously. Alternately, multiple samples can be sequentially drawn into the microfluidic device and routed internally to multiple channels for simultaneous processing and analysis. Sample interfaces with dried samples can also be performed using this basic system, e.g., by partly or completely expelling fluid from the capillary to hydrate samples prior to drawing them into the microfluidic device (the fluid is typically contacted to the samples as a hanging drop on the tip of the capillary and then drawn back into the capillary). For either approach, see also, U.S. Patent Nos. 6,482,364, 6,042,709, 6,287,520 and 6,235,471. Essentially any fluid manipulation (aliquotting, diluting, heating and cooling) can be performed in the network using available methods. Details regarding dilution and aliquotting operations in microscale devices can be found in the patent literature, e.g., U.S. Patent Nos. 6,149,870, 5,869,004 and 6,440,722. Samples and components to be mixed/diluted or aliquotted can be brought into the microscale device through pipettor elements or from reaction component

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reservoirs on the device itself, or, commonly, both. For example, the sample can be brought into the microfluidic device through a pipettor channel and diluted and supplied with common reagents from an on device dilution and/or reagent reservoir(s). Locus specific reagents (e.g., amplification primer pairs) can be on the device in wells, or stored off the device, e.g., in microtiter plates (in which case they can be accessed by the pipettor channel). Any or all of these operations can be performed in a continuous or stopped flow format.

[0090] The microfluidic device typically performs several functions, including reaction assembly (assembly of reaction mixtures), thermocycling, and acting as a "cuvette" for an optical system during an imaging (detection) step. In the reaction assembly, the reaction mixture components (particularly magnesium and the enzyme) which get combined at the last second before heating begins are assembled. This is called a "hot start" and provides advantages of specificity. During thermocycling, the system optionally provides both constant fluid movement and a continuous sequence of temperature changes. During imaging, a high data rate CCD detector, a multiple-pixel array detector or a fiber-optic ball head lens detector, for example, is useful in providing an adequate dynamic range for methods of quantification.

[0091] Commercial systems that perform all aspects of fluid handling that can be used in the practice of the present invention are available. Examples include the 250 HTS system and AMS 90 SE from Caliper Life Sciences (Hopkinton, MA). These systems perform experiments in serial, continuous flow fashion and employ a "chip-to-world" interface, or sample access system, called a sipper through which materials in microwell plates are sipped into a capillary or capillaries attached to the chip and drawn into the channels of the chip. There they are mixed with components of interest and a processing and result detection steps are performed. See, for example, U.S. Published Patent Application No. 2005/0042839.

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[0092] Whether conventional fluid handling or microfluidic approaches (or both) are used, the aliquotting and/or dilution events can be performed to achieve particular results. For example, a sample can be diluted equally in each aliquot, or, alternately, the aliquots can be differentially diluted (e.g., a dilution series can be made). The aliquots themselves can be of a volume that is appropriate to the fluid handling approach being used by the system, e.g., on the order of a few microliters for microtiter plates to 100 nL, 10 nL or even 1 nL or less for microfluidic approaches.

[0093] The aliquots can be selected to have high or low copy numbers of any relevant nucleic acid (e.g., for low copy number aliquots, 50 or fewer, generally 25 or fewer, usually 10 or fewer and often 5 or fewer, 2 or fewer or 1 or fewer copies of the relevant nucleic acid(s)). The number of aliquots generated will depend on the size of the sample and the amount of quantitative information desired by the practitioner. For example, where simple detection of a rare nucleic acid is desired, enough low and/or single copy number aliquots are made of the sample to detect the nucleic acid in one of the aliquots. Where more quantitative information is needed, enough copies are made to provide reliable statistical information, e.g., to a given confidence value. In either case, this can include anywhere from 1 aliquot to 10.sup.9 or more aliquots, e.g., 10, 100, 1,000, 10,000, 100,000, 1,000,000, 1,000,000,000 or more aliquots. There is no theoretical limit on the number of aliquots that can be made and assessed for a nucleic acid of interest according to the present invention, though there are practical considerations with respect to the throughput of the system and the size of the sample (the lower the throughput, the fewer aliquots can be analyzed in a given time; the larger the sample size the more aliquots can be made of the sample). Using microfluidic approaches, reagent usage (and concomitant reagent costs) can be minimized. By formatting the system to provide for continuous flow of sample and

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reagents, including, optionally, during amplification, the systems of the invention can greatly speed the process of searching many different samples for a nucleic acid of interest. Similarly, if stopped flow approaches are used, simultaneous processing of signals from PCR reactions can be used to speed the process of searching samples for a nucleic acid of interest. In the examples below, about 150 aliquots for each dilution range was sufficient to provide reasonable quantitative information for Poisson statistics for model samples. Obviously, more or fewer aliquots can be used in the methods as well.

[0094] The methods of the invention include amplifying one or more sequences of a nucleic acid of interest from a sample or aliquot and, optionally, one or more additional nucleic acids. Real time PCR and/or real time reverse transcriptase-PCR (e.g., mediated via TaqMan® probes, molecular beacon-based probes or quantum dot based probes) are used to facilitate detection of amplified nucleic acids.

[0095] It is expected that one of skill is generally familiar with the details of these amplification methods. Details regarding these amplification methods can be found, e.g., in Sambrook and Russell, *Molecular Cloning*, 3rd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001); Ausubel et al., *Current Protocols in Molecular Biology* (John Wiley & Sons, New York, updated through 2005); *Real-Time PCR: An Essential Guide*, K. Edwards et al., eds. (Horizon Bioscience, Norwich, U.K., 2004).

[0096] In one aspect, real time PCR is performed on the various aliquots or reaction mixtures such as described herein, e.g., using fluorescent labels. It will be recognized that fluorescent labels are not to be limited to single species organic molecules, but include inorganic molecules, multi-molecular mixtures of organic and/or inorganic molecules, crystals, heteropolymers, and the like. Suitable fluorescent labels can be provided by, e.g., DNA binding dyes, molecular

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beacons, TaqMan® probes or quantum dots. The amplification product (which is double stranded) binds dye molecules in solution to form a complex. With the appropriate dyes, it is possible to distinguish between dye molecules free in solution and dye molecules bound to amplification product. For example, certain dyes fluoresce only when bound to amplification product. Examples of suitable dyes include, but are not limited to, LC Green (Idaho Technology, Salt Lake City, Utah), SYBR® Green, SYBR® GreenER™ and Pico Green (Invitrogen Corp., Carlsbad, Calif.), ethidium bromide, propidium iodide, chromomycin, acridine orange, Hoechst 33258, Hoechst 33342, Toto-1, Yoyo-1, and DAPI (4',6-diamidino-2-phenylindole hydrochloride). Additional discussion regarding the use of intercalation dyes is provided by Zhu et al. (*Anal Chem* **66**:1941-1948, 1994).

[0097] A molecular beacon (MB) is an oligonucleotide or PNA which, under appropriate hybridization conditions, self-hybridizes to form a stem and loop structure. The MB has a label and a quencher at the termini of the oligonucleotide or PNA; thus, under conditions that permit intra-molecular hybridization, the label is typically quenched (or at least altered in its fluorescence) by the quencher. Under conditions where the MB does not display intra-molecular hybridization (e.g., when bound to a target nucleic acid, e.g., to a region of an amplicon during amplification), the MB label is unquenched.

[0098] Details regarding standard methods of making and using MBs are well established in the literature and MBs are available from a number of commercial reagent sources. See also, e.g., Leone et al. (*Nucl Acids Res* **26**:2150-2155, 1995); Tyagi and Kramer (*Nat Biotechnology* **14**:303-308, 1996); Blok and Kramer (*Mol Cell Probes* **11**:187-194, 1997); Hsuih et al. (*J Clin Microbiol* **34**:501-507, 1997); Kostrikis et al. (*Science* **279**:1228-1229, 1998); Sokol et al. (*Proc Natl Acad Sci. USA* **95**:11538-11543, 1998); Tyagi et al. (*Nat Biotechnology* **16**:49-53, 1998;

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Bonnet et al. (*Proc Natl Acad Sci USA* **96**:6171-6176, 1999); Fang et al. (*J Am Chem Soc* **121**:2921-2922, 1999); Marras et al. (*Genet Anal Biomol Eng* **14**:151-156, 1999); and Vet et al. (*Proc Natl Acad Sci USA* **96**:6394-6399, 1999). Additional details regarding MB construction and use is found in the patent literature, e.g., U.S. Patent Nos. 5,925,517, 6,150,097 and 6,037,130.

[0099] MBs are robust reagents for detecting and quantifying nucleic acids, including in real time, e.g., during PCR, LCR or other nucleic acid amplification reactions (e.g., MBs can be used to detect targets as they are formed). A variety of commercial suppliers produce standard and custom molecular beacons, including Cruachem (cruachem.com), Oswel Research Products Ltd. (UK; oswel.com), Invitrogen Corp. (Carlsbad, Calif.; invitrogen.com), the Midland Certified Reagent Company (Midland, Tex. mcrc.com) and Gorilla Genomics, LLC (Alameda, Calif.). A variety of kits which utilize molecular beacons are also commercially available, such as the Sentinel™. Molecular Beacon Allelic Discrimination Kits from Stratagene (La Jolla, Calif.) and various kits from Eurogentec SA (Belgium, eurogentec.com) and Isogen Bioscience BV (The Netherlands, isogen.com).

[0100] MB components (e.g., oligos, including those labeled with fluorophores or quenchers) can be synthesized using conventional methods. For example, oligos or peptide nucleic acids (PNAs) can be synthesized on commercially available automated oligonucleotide/PNA synthesis machines using standard methods. Labels can be attached to the oligos or PNAs either during automated synthesis or by post-synthetic reactions which have been described before see, e.g., Tyagi and Kramer (*Nat Biotechnology* **14**:303-308, 1996) and U.S. Pat. Nos. 6,037,130 and 5,925,517. Additional details on synthesis of functionalized oligos can be found in Nelson et al. *Nucl Acids Res* **17**:7187-7194, 1989). Labels/quenchers can be introduced to the

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oligonucleotides or PNAs, e.g., by using a controlled-pore glass column to introduce, e.g., the quencher (e.g., a 4-dimethylaminoazobenzene-4'-sulfonyl moiety (DABSYL). For example, the quencher can be added at the 3' end of oligonucleotides during automated synthesis; a succinimidyl ester of 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL) can be used when the site of attachment is a primary amino group; and 4-dimethylaminophenylazo- phenyl-4'-maleimide (DABMI) can be used when the site of attachment is a sulphydryl group. Similarly, fluorescein can be introduced in the oligos, either using a fluorescein phosphoramadite that replaces a nucleoside with fluorescein, or by using a fluorescein dT phosphoramadite that introduces a fluorescein moiety at a thymidine ring via a spacer. To link a fluorescein moiety to a terminal location, iodoacetoamidofluorescein can be coupled to a sulphydryl group. Tetrachlorofluorescein (TET) can be introduced during automated synthesis using a 5'-tetrachloro-fluorescein phosphoramadite. Other reactive fluorophore derivatives and their respective sites of attachment include the succinimidyl ester of 5-carboxyrhodamine-6G (RHD) coupled to an amino group; an iodoacetamide of tetramethylrhodamine coupled to a sulphydryl group; an isothiocyanate of tetramethylrhodamine coupled to an amino group; or a sulfonylchloride of Texas red coupled to a sulphydryl group. During the synthesis of these labeled components, conjugated oligonucleotides or PNAs can be purified, if desired, e.g., by high pressure liquid chromatography or other methods.

[0101] TaqMan® probes are composed of short (e.g., 20-25 bases) oligodeoxynucleotides that are labeled with two different fluorescent dyes. On the 5' terminus of each probe is a reporter dye, and on the 3' terminus of each probe a quenching dye is found. The oligonucleotide probe sequence can be complementary to an internal target sequence present in a PCR amplicon. When the probe is intact, energy transfer occurs between the two fluorophores and emission

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from the reporter is quenched by the quencher (fluorescent resonant energy transfer or FRET). During the extension phase of PCR, the probe is cleaved by 5' nuclease activity of the polymerase used in the reaction, thereby releasing the reporter from the oligonucleotide-quencher and producing an increase in reporter emission intensity.

[0102] Accordingly, TaqMan® probes are oligonucleotides that have a label and a quencher, where the label is released after hybridization and during amplification by the exonuclease action of the polymerase used in amplification. This provides a real time measure of amplification during synthesis. A variety of TaqMan® reagents are commercially available, e.g., from Applied Biosystems (Division Headquarters in Foster City, Calif.) as well as from a variety of specialty vendors such as Biosearch Technologies (e.g., black hole quencher probes).

[0103] A quantum dot is a fluorescent semiconductor nanocrystal having a characteristic spectral emission, which is tunable to a desired energy by selection of the particle size of the quantum dot. The quantum dot emits a characteristic emission spectrum which can be observed and measured spectrophotometrically. The emission spectra of quantum dots have linewidths as narrow as 25-30 nm depending on the size heterogeneity of the sample, and lineshapes that are symmetric, gaussian or nearly gaussian with an absence of a tailing region. The combination of tunability, narrow linewidths, and symmetric emission spectra without a tailing region provides for high resolution of multiply-sized quantum dots within a system and enables researchers to examine simultaneously a variety of biological moieties tagged with quantum dots. In addition, the range of excitation wavelengths of the nanocrystal quantum dots is broad and can be higher in energy than the emission wavelengths of all available quantum dots. Consequently, this allows the simultaneous excitation of all quantum dots in a system with a single light source, usually in the ultraviolet or blue region of the spectrum. Quantum dots are also more robust than

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conventional organic fluorescent dyes and are more resistant to photobleaching than the organic dyes. The use of quantum dots as fluorescent labels of nucleotides is described in Bruchez et al. (*Science* **281**:2013-2016, 1998), Warren and Nie (*Science* **281**:2016-2018, 1998), Alivisatos (*Science* **271**:933-937, 1999) and U.S. Patent Nos. 6,544,732 and 6,855,551. A variety of quantum dot reagents are commercially available, e.g., from Invitrogen Corp. (Carlsbad, Calif.; invitrogen.com) or Evident Technologies (Troy, NY, evidenttech.com).

[0104] Multicolor, multiplexed assays are a particular strength of quantum dot bioconjugates. The emission from quantum dot nanocrystals is narrow and symmetric; therefore, overlap with other colors is minimal, yielding less bleed through into adjacent detection channels and attenuated crosstalk and allowing many more colors to be used simultaneously. Since each bioconjugate color is based upon the same underlying material (they differ only in size), the conjugation and use methods for one color are easily extrapolated to all of the different colors, simplifying and speeding assay development. Furthermore, every quantum dot nanocrystal can be excited using a single light source -- narrow laser and broad lamp excitation are both useful. Three- or four-color detection no longer requires multiple lasers or laborious alignments and compensations.

[0105] In general, synthetic methods for making oligonucleotides, including probes, molecular beacons, PNAs, LNAs (locked nucleic acids), etc., are well known. For example, oligonucleotides can be synthesized chemically according to the solid phase phosphoramidite triester method described by Beaucage and Caruthers (*Tetrahedron Letts* **22**:1859-1862, 1981), e.g., using a commercially available automated synthesizer, e.g., as described in Needham-VanDevanter et al. (*Nucl Acids Res* **12**:6159-6168, 1984). Oligonucleotides, including modified oligonucleotides can also be ordered from a variety of commercial sources known to persons of

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skill. There are many commercial providers of oligo synthesis services, and thus this is a broadly accessible technology. Any nucleic acid can be custom ordered from any of a variety of commercial sources, such as The Midland Certified Reagent Company (mcrc@oligos.com), The Great American Gene Company (www.genco.com), ExpressGen Inc. (www.expressgen.com), Operon Technologies Inc. (Alameda, Calif.) and many others. Similarly, PNAs can be custom ordered from any of a variety of sources, such as PeptidoGenic (pkim@ccnet.com), HTI Bio-products, inc. (www.htibio.com), BMA Biomedicals Ltd (U.K.), Bio-Synthesis, Inc., and many others.

[0106] A number of high throughput approaches to performing PCR and other amplification reactions have been developed, e.g., involving amplification reactions in microfluidic devices, as well as methods for detecting and analyzing amplified nucleic acids in or on the devices. These high throughput approaches can be adapted for use with the present invention. Details regarding such technology is found, e.g., in the technical and patent literature, e.g., Kopp et al. (*Science* **280**:1046-1048, 1998), Chow et al. (Science 282:396-399, 1998), Zhang et al. (*Anal Chem* **71**:1138-1145, 1999), U.S. Patent Nos. 6,444,461, 6,406,893, 6,391,622, 6,303,343, 6,171,850, 5,939,291, to 5,955,029, 5,965,410 and 7,015,030, U.S. Published Patent Application Nos. 2004/0180346 and 2005/0042639, and many others.

[0107] Any available method for detecting amplified nucleic acids can be used in the present invention. Common approaches include real time amplification detection with molecular beacons, quantum dots or TaqMan® probes, detection of intercalating dyes (such as those described above), detection of labels incorporated into the amplified nucleic acids themselves and the like. Amplified nucleic acids (amplicons) can be detected in homogenous (substantially unseparated) reaction mixtures or solutions.

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[0108] Amplification and detection are commonly integrated in a system comprising a microfluidic device in the present invention. Available microfluidic systems that include detection features for detecting nucleic acids include the 250 HTS system and AMS 90 SE from Caliper Technologies (Mountain View, Calif.), as well as the Agilent 2100 bioanalyzer (Agilent, Palo Alto, Calif.). Additional details regarding systems that comprise detection (and separation/detection) capabilities are well described in the patent literature, e.g., the references described herein and in PCT Published Patent Application No. WO 98/00231.

[0109] In general, the devices herein optionally include signal detectors, e.g., which detect fluorescence, phosphorescence, radioactivity, pH, charge, absorbance, luminescence, temperature, magnetism or the like. Fluorescent detection is especially preferred and generally used for detection of amplified nucleic acids, particularly for real-time PCR amplification (however, downstream operations can be performed on amplicons, which can involve other detection methods, such as mass spectroscopy or size exclusion).

[0110] The detector(s) optionally monitor one or a plurality of signals from an amplification reaction and/or hybridization reaction. For example, the detector can monitor optical signals which correspond to "real time" amplification assay results. The detector can monitor a single type of signal, or, e.g., simultaneously monitor multiple different signals.

[0111] Example detectors include photomultiplier tubes, photodiodes, avalanche photodiodes, photoresistors, bolometers, microchannel plate detectors, CCD arrays (including intensified and electron multiplying CCD arrays), CMOS image sensors, and/or the like. Wavelength discrimination may be achieved through the use of multilayer dielectric interference filters, color absorptive filters, or by dispersion with diffractive and/or refractive optical elements. Amplicons or other components which emit a detectable signal can be flowed past the detector, or,

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alternatively, the detector can move relative to the site of the amplification reaction (or, the detector can simultaneously monitor a number of spatial positions corresponding to channel regions, or microtiter wells e.g., as in a CCD array). Detectors in the present invention can detect signals from probes associated with nucleic acids of the invention that flow into one or more detection regions, e.g., of a microfluidic device.

[0112] The detector can include or be operably linked to a computer (or other logic device), e.g., which has software for converting detector signal information into assay result information (e.g., presence of a nucleic acid of interest, the length of a nucleic acid of interest, proportions of nucleic acid of interest lengths, and/or correlations with disease states), or the like.

[0113] Signals are optionally calibrated, e.g., by calibrating the microfluidic system by monitoring a signal from a known source. For example, signals can be calibrated against a reference light source, internal reference signals, or normalized for detection of positive signals over background.

[0114] A microfluidic system according to the present invention can also employ multiple different detection systems for monitoring signals in the system. Detection systems of the present invention are used to detect and monitor the materials in a particular channel region (or other reaction detection region). Once detected, the flow rate and velocity of any cells or droplets in the channels can be optionally measured by sensors and controlled as described above.

[0115] Examples of detection systems useful in methods and systems of the invention can include optical sensors, temperature sensors, pressure sensors, pH sensors, conductivity sensors, and the like. Each of these types of sensors is readily incorporated into the microfluidic systems described herein. In these systems, such detectors can be placed either within or adjacent to the

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microfluidic device or one or more channels, chambers or conduits of the device, such that the detector is within sensory communication with the device, channel, or chamber. The phrase "within sensory communication" of a particular region or element, as used herein, generally refers to the placement of the detector in a position such that the detector is capable of detecting the property of the microfluidic device, a portion of the microfluidic device, or the contents of a portion of the microfluidic device, for which that detector was intended. For example, a pH sensor placed in sensory communication with a microscale channel is capable of determining the pH of a fluid disposed in that channel. Similarly, a temperature sensor placed in sensory communication with the body of a microfluidic device is capable of determining the temperature of the device itself.

[0116] Preferred detection systems in accordance with the present invention include optical detection systems for detecting an optical property of a material within the channels and/or chambers of the microfluidic devices that are incorporated into the microfluidic systems described herein. Such optical detection systems are typically placed adjacent to a microscale channel of a microfluidic device, and are in sensory communication with the channel via an optical detection window that is disposed across the channel or chamber of the device. Optical detection systems include systems that are capable of measuring the light emitted from material within the channel, the transmissivity or absorbance of the material, as well as the materials spectral characteristics. In preferred aspects, the detector measures an amount of light emitted from the material, such as a fluorescent or chemiluminescent material. As such, the detection system will typically include collection optics for gathering a light based signal transmitted through the detection window, and transmitting that signal to an appropriate light detector. Microscope objectives of varying power, field diameter, and focal length are readily utilized as at

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least a portion of this optical train. The light detectors are optionally spectrophotometers, photodiodes, avalanche photodiodes, photomultiplier tubes, diode arrays, or in some cases, imaging systems, such as charged coupled devices (CCDs) and the like. The detection system is typically coupled to a computer, via an analog to digital or digital to analog converter, for transmitting detected light data to the computer for analysis, storage and data manipulation.

[0117] In the case of fluorescent materials such as labeled amplicons, the detector typically includes a light source that produces light at an appropriate wavelength for activating the fluorescent material, as well as optics for directing the light source through the detection window to the material contained in the channel or chamber. The light source can be any number of light sources that provides an appropriate wavelength, including lasers and LEDs. Other light sources are used in other detection systems. For example, broad band light sources are typically used in light scattering/transmissivity detection schemes, and the like. Typically, light selection parameters are well known to those of skill in the art.

[0118] The detector can exist as a separate unit, but can also be integrated with the system or microfluidic device, into a single instrument. Integration of these functions into a single unit facilitates connection of these instruments with the computer, by permitting the use of few or a single communication port(s) for transmitting information between the controller, the detector and the computer.

[0119] General references that are useful in understanding how to generate and analyze data, as well as other relevant concepts include: Weiss (*Introductory Statistics*, 7th Ed., Addison-Wesley, Reading, Mass., 2004); Weiss (*Elementary Statistics* , 5th Ed., Addison-Wesley, Reading, Mass., 2001); Berinstein (*Finding Statistics Online: How to Locate the Elusive Numbers You Need*, Information Today, Medford, N.J., 1998); Everitt, (*The Cambridge*

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Dictionary of Statistics, Cambridge University Press, New York, 1998); Kotz (*Encyclopedia of Statistical Sciences*, vol. 1-9 plus supplements, Wiley, New York, 1988); Dillon and Goldstein (*Multivariate Analysis: Methods and Applications*, Wiley, New York, 1984); Tabachnick and Fidell (*Using Multivariate Statistics*, HarperCollins College Publishers, New York, 1996); Box et al. (*Statistics for Experimenters*, Wiley, New York, 1978); Cornell (*Experiments with Mixtures*, Wiley, New York, 1990); John (*Statistical Design and Analysis of Experiments*, SIAM, Philadelphia, 1998); Gibas and Jambeck (*Bioinformatics Computer Skills*,O'Reilly, Sebastipol, Calif., 2001); Pevzner (*Computational Molecular Biology and Algorithmic Approach*, The MIT Press, Cambridge, Mass., 2000); Durbin et al. (*Biological Sequence Analysis: Probabilistic Models of Proteins and Nucleic Acids*, Cambridge University Press, Cambridge, UK, 1998); and Rashidi and Buehler (*Bioinformatic Basics: Applications in Biological Science and Medicine*, CRC Press LLC, Boca Raton, Fla., 2000).

[0120] The highly reproducible peak parameters, e.g., amplitude, width area, and/or shape features of a signal from an amplification reaction can be correlated to the starting copy number for the reaction and/or used to discriminate signals of interest from background fluctuations. This correlation can be performed at the theoretical level, taking thermal diffusivity and Taylor Aris dispersion into account, or it can be performed by comparison to standards (e.g., comparisons to peak shapes, e.g., heights, widths, or general shape profiles for amplification reactions that have known copy numbers for starting materials). The same or different peak parameters can be evaluated in interpretation of detector signals for two on more probes in determination of nucleic acid length.

[0121] For freely diffusing particles in a pressure-driven, laminar flow channel, the concentration profile along the channel may be approximated using a modified diffusion

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equation. See G.I. Taylor (*Proc Roy Soc Lond* A **219**:186(1953)) and R. Aris (*Proc Roy Soc Lond* A **235**:67 (1956)). The Taylor dispersion coefficient (D_T) is dependent on the dimensions and shape of the microfluidic cavity through which the marker is flowed, the average flow velocity (v) and the molecular diffusivity (D_m). $D_T = D_m (1 + k\, v^2 w^2 / D_m^2)$, where w is a characteristic channel width and k is a dimensionless factor that depends on the shape of the channel cross-section. Preferably, fluid parameters and channel dimensions are chosen to reduce dispersion to an acceptable level.

[0122] The systems of the invention can include microfluidic devices, reaction mixtures, detectors, sample storage elements (microtiter plates, dried arrays of components, etc.), flow controllers, amplification devices or microfluidic modules, computers and/or the like. These systems can be used for aliquotting, amplifying and analyzing the nucleic acids of interest. The microfluidic devices, amplification components, detectors and storage elements of the systems have already been described in some detail above. The following discussion describes appropriate controllers and computers, though many configurations are available and one of skill would be expected to be familiar in their use and would understand how they can be applied to the present invention.

[0123] A variety of controlling instrumentation is optionally utilized in conjunction with the microfluidic devices described herein, for controlling the transport and direction of fluids and/or materials within the devices of the present invention, e.g., by pressure-based or electrokinetic control.

[0124] For example, in many cases, fluid transport and direction are controlled in whole or in part, using pressure based flow systems that incorporate external or internal pressure sources to drive fluid flow. Internal sources include microfabricated pumps, e.g., diaphragm pumps,

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thermal pumps, Lamb wave pumps and the like that have been described in the art. See, e.g., U.S. Pat. Nos. 5,271,724, 5,277,556, and 5,375,979 and PCT Published Patent Application Nos. WO 94/05414 and WO 97/02357. The systems described herein can also utilize electrokinetic material direction and transport systems.

[0125] Preferably, external pressure sources are used, and applied to ports at channel termini. These applied pressures, or vacuums, generate pressure differentials across the lengths of channels to drive fluid flow through them. In the interconnected channel networks described herein, differential flow rates on volumes are optionally accomplished by applying different pressures or vacuums at multiple ports, or preferably, by applying a single vacuum at a common waste port and configuring the various channels with appropriate resistance to yield desired flow rates. Example systems are described in U.S. Published Patent Application No. 2002/0019059.

[0126] Typically, the controller systems are appropriately configured to receive or interface with a microfluidic device or system element as described herein. For example, the controller and/or detector, optionally includes a stage upon which a microfluidic device is mounted to facilitate appropriate interfacing between the controller and/or detector and the device. Typically, the stage includes an appropriate mounting/alignment structural element, such as a nesting well, alignment pins and/or holes, asymmetric edge structures (to facilitate proper device alignment), and the like. Many such configurations are described in the references cited herein.

[0127] The controlling instrumentation discussed above is also optionally used to provide for electrokinetic injection or withdrawal of material downstream of the region of interest to control an upstream flow rate. The same instrumentation and techniques described above are also utilized to inject a fluid into a downstream port to function as a flow control element.

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[0128] As noted above, either or both of the controller system and/or the detection system can be coupled to an appropriately programmed processor or computer (logic device) which functions to instruct the operation of these instruments in accordance with preprogrammed or user input instructions, receive data and information from these instruments, and interpret, manipulate and report this information to the user. As such, the computer is typically appropriately coupled to one or both of these instruments (e.g., including an analog to digital or digital to analog converter as needed), such as the main control and processing computer 105 illustrated in Figure 1.

[0129] The computer typically includes appropriate software for receiving user instructions, either in the form of user input into a set parameter fields, e.g., in a GUI, or in the form of preprogrammed instructions, e.g., preprogrammed for a variety of different specific operations. The software then converts these instructions to appropriate language for instructing the operation of the fluid direction and transport controller to carry out the desired operation. The computer then receives the data from the one or more sensors/detectors included within the system, and interprets the data, either provides it in a user understood format, or uses that data to initiate further controller instructions, in accordance with the programming, e.g., such as in monitoring and control of flow rates (including for continuous flow), temperatures, applied voltages, and the like.

[0130] The systems and/or kits can include system instructions (e.g., embodied in a computer or in a computer readable medium, e.g., as system software) for practicing any of the method steps herein. For example, the system optionally includes system software that correlates a shape, length, width, volume and/or area occupied by amplified copies of the nucleic acid of interest, as detected by the detector, to the number of copies of the nucleic acid of interest

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present in one of the aliquots, or to the number of copies of the nucleic acid of interest present in the sample, or both. Similarly, the system optionally includes system instructions that direct the dilution module to aliquot the sample into a plurality of aliquots, including a plurality of zero copy aliquots comprising no copies of the nucleic acids of interest and one or more single copy aliquot comprising a single copy of the nucleic acid of interest.

[0131] The statistical functions noted above can also be incorporated into system software, e.g., embodied in the computer, in computer memory or on computer readable media. For example, the computer can include statistical or probabilistic system software that performs one or more statistical or probabilistic analysis of signals received from one or more of the aliquots subjected to amplification (e.g., via thermocycling). The statistical or probabilistic analysis software optionally quantitatively determines a concentration, proportion, or number of the nucleic acids of interest in the sample.

[0132] Computers and software of the systems receive and evaluate signal data from one or more analyses to provide quantitation and/or proportionality determinations for nucleic acids of interest. In a basic form, e.g., the amplitude or integrated area of a signal can be adjusted with a conversion factor for an output in desired units, such as, e.g., copies per nL, ng/μL, and the like. Alternately, one or more standard materials of known concentration can be analyzed to provide data for regression analyses wherein changes in detectable signals with changes in concentration are expressed as an equation (standard curve) from which unknown concentrations can be determined by insertion of one or more signal parameters into the equation. In a particular embodiment, quantitation of a nucleic acid of interest can be based on the number of amplification cycles required to obtain a signal of a certain intensity.

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[0133] In the present invention, the computer typically includes software for the monitoring of materials in the channels. Additionally, the software is optionally used to control electrokinetic or pressure modulated injection or withdrawal of material. The injection or withdrawal is used to modulate the flow rate as described above, to mix components, and the like.

[0134] The following example is offered to illustrate, but not to limit the claimed invention. It is understood that the example and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

[0135] An experiment was conducted to demonstrate the real-time detection of PCR amplification in a microfluidic device under continuous flow conditions. An 8 channel microfluidic device was used for this experiment. The dimensions of each microchannel were approximately 180 μm wide by 11 μm deep. The reaction zone of the microfluidic device was approximately 40 mm in length. fimA bacterial DNA was amplified using standard real-time PCR techniques. Test solution containing fimA DNA template and real-time PCR reagents was alternated with carrier fluid that optionally contained a flow marker. A 60 sec PCR cycle time was used including 10 sec at 95° C, 10 sec at 55° C and 20 sec at 72° C per cycle. The flow rate was approximately 1 mm per min with continuous flow in the microchannels during the experiment. The real-time PCR system used in this experiment included an LED array with a 470 nm peak and a 630 nm peak an excitation filter and a dual bandpass interference filter. The detector conditions were as follows: dectector: CMOS 12.8 Mpixel; lens: 50 mm f/1.4; magnification: approximately 1:1; emission filter: interference filter with passbands of 510 -565

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nm and 660-710nm; ISO sensitivity: 3200. Simultaneous multi-color image acquisition was performed during the 72°C extension phase. SYBR Green dye was used to detect amplified product, and AlexaFluor 647 red dye was optionally used as a flow marker.

[0136] The results of this experiment are shown in Figures 8A-8B and Figures 9A-9B. Figure 8A shows a 30 mm long section of the channels under continuous-flow PCR conditions. Red flow markers consisting of Alexa Fluor 647 dye boluses are visible. The green light, noticcable only at the downstream end of the channel on the right, is from SYBR Green I dye indicating the presence of amplified DNA. Figure 8B shows a plot of the intensity of the red signal along microchannel 2 (second from the top) from Figure 8A. Diffusion and dispersion cause these dye marker boluses to spread as they move down the channel, resulting in broader and shorter signal peaks downstream. Nevertheless, these effects are weak and the boluses retain sufficient distinctness so that they can be used to measure the flow speed.

[0137] Figure 9A shows a 30 mm long section of the channels under continuous-flow PCR conditions. The green light is from SYBR Green dye fluorescence acquired during the extension phase and indicates the presence of amplified DNA. In this case, no separate flow markers were injected into the channels. Figure 9B shows a plot of the intensity of the green signal along microchannel 2 (second from the top) from Figure 9A. Toward the upstream end of the channel, where sample elements have experienced few PCR temperature cycles, the measured intensity is dominated by background scattering. Going downstream, the signal rises steeply and then saturates. This sigmoidal shape of signal versus position is similar to the shape of signal amplitude versus cycle number seen in conventional real-time PCR systems. These results show real-time detection of PCR amplification under continuous flow conditions.

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[0138] The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0139] Embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

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[0140] All publications, patents, patent applications, and/or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, and/or other document were individually indicated to be incorporated by reference for all purposes.

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WHAT IS CLAIMED IS:

1. A method of performing real-time PCR comprising the steps of:

a) continuously moving a bolus of test solution containing real-time PCR reagents in a channel;

b) moving a carrier fluid in said channel, sequentially alternating with a test bolus;

c) cycling the temperature in a defined section of the channel in order to achieve PCR; and

d) measuring the intensity of the fluorescent signal at a plurality of locations along said defined section of said channel.

2. The method of claim 1, which further comprises the step of measuring the average flow speed in the channel.

3. The method of claim 2, wherein the average flow speed is measured by comparing sequential images of the fluorescent signal from the channel.

4. The method of claim 2, wherein the average flow speed is measured by comparing sequential images of a marker in the channel.

5. The method of claim 4, wherein the marker is in the test solution.

6. The method of claim 4, wherein the marker is in the carrier fluid.

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7. The method of claim 2, which further comprises the step of adjusting the flow rate to control the timing of each PCR cycle.

8. The method of claim 7, wherein the flow rate is adjusted by adjusting the pressure at an inlet or outlet of the channel.

9. The method of claim 1, wherein the intensity of the fluorescent signal is measured at least once during each PCR cycle.

10. The method of claim 2, wherein the intensity of the fluorescent signal is measured at least once during each PCR cycle.

11. The method of claim 7, wherein the intensity of the fluorescent signal is measured at least once during each PCR cycle.

12. The method of claim 7, wherein the average flow speed is measured by comparing sequential images of the fluorescent signal from the channel.

13. The method of claim 7, wherein the average flow speed is measured by comparing sequential images of a marker in the channel.

14. The method of claim 13, wherein the marker is in the test solution.

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15. The method of claim 13, wherein the marker is in the carrier fluid.

16. The method of claim 1, wherein the step of cycling the temperature further includes the steps of:

i) heating said defined section of the channel to a first temperature;

ii) cooling said defined section of the channel to a second temperature; and

iii) heating said defined section of the channel to a third temperature.

17. The method of claim 16, wherein the first temperature is in the range of about 85° C to about 100° C, the second temperature is in the range of about 20° C to about 70° C and the third temperature is in the range of about 55° C to about 80° C.

18. The method of claim 1, wherein said defined section of the channel has a length suitable for completing 10 to 50 cycles of PCR.

19. A method for monitoring the progress of a polymerase chain reaction in a channel, comprising the steps of:

a) moving a bolus of test solution containing real-time PCR reagents in a channel;

b) moving a carrier fluid in said channel, sequentially alternating with a test bolus;

c) cycling the temperature in a defined section of the channel in order to achieve PCR;

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d) capturing an image of a reaction-dependent fluorescence signal along a section of the channel;

e) measuring the average flow speed in the channel; and

f) relating position of the test bolus to the number of temperature cycles experienced by a test bolus from the average flow speed.

20. The method as in claim 19, wherein the average flow speed is measured by comparing sequential images of the reaction-dependent fluorescent signal from the channel.

21. The method as in claim 19, wherein the average flow speed is measured by comparing sequential images of a reaction-independent flow marker from the channel.

22. The method as in claim 21, wherein the reaction-independent flow marker is pre-mixed in the test bolus.

23. The method as in claim 21, wherein the reaction-independent flow marker is introduced to the channel as a bolus alternating with the test bolus.

24. The method as in claim 21, wherein the reaction-independent flow marker is pre-mixed in the carrier fluid.

25. The method as in claim 21, wherein scattered light from the reaction-independent flow marker is resolvable from the reaction-dependent fluorescence by wavelength spectrum.

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26. The method as in claim 21, wherein scattered light from the reaction-independent flow marker is resolvable from the reaction-dependent fluorescence on the basis of fluorescence lifetime.

27. The method as in claim 21, wherein the reaction-independent flow marker is further used to determine the flow dispersion of the test bolus.

28. The method as in claim 19, wherein the image of reaction-dependent fluorescence is captured at least once per PCR cycle.

29. The method as in claim 19, wherein the image of reaction-dependent fluorescence is captured sequentially by scanning a length of the channel on a time scale shorter than the duration of one PCR cycle.

30. The method as in claim 19, wherein the image of reaction-dependent fluorescence is captured by acquiring signal from multiple points along the channel simultaneously.

31. The method as in claim 19, wherein the flow rate measurements are part of a feedback loop for regulating the flow rate.

32. The method as in claim 19, wherein the flow speed is measured through detecting a sample bolus entrance into and exit from a defined section of the channel.

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33. A method for monitoring the progress of a polymerase chain reaction in a channel, comprising the steps of:

a) moving a bolus of test solution containing real-time PCR reagents in a channel;

b) moving a carrier fluid in the same channel, sequentially alternating with a test bolus;

c) applying spatial temperature zones along the channel to achieve PCR;

d) monitoring the fluorescence signal at fixed spatial locations along the channel corresponding to fixed points in the PCR cycle;

e) measuring the average flow speed of the fluid in the channel; and

f) adjusting the flow rate in order to control the timing of the PCR cycle.

34. A system for performing real-time PCR in a microfluidic device, said system comprising:

a microfluidic device comprising at least one channel configured to contain at least one test solution containing real-time PCR reagents and at least one carrier fluid;

a thermal transfer element integral with or proximal to the microfluidic device, said thermal transfer element configured to thermocycle the test solution along a defined section of the at least one channel for performing real-time PCR in the test solution;

a source of illumination integral with or proximal to the microfluidic device and configured to illuminate the at least one channel; and

a detector integral with or proximal to the microfluidic device, said detector configured to detect a fluorescent signal at a plurality of locations along said defined section of said at least one channel.

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35. The system of claim 34, further comprising a software system that measures an average flow speed of the test solution in the channel.

36. The system of claim 35, wherein said software system measures the average flow speed of the test solution by comparing sequential images of the fluorescent signal in the channel.

37. The system of claim 35, wherein said software system measures the average flow speed of the test solution by comparing sequential images of a marker in the channel.

38. The system of claim 37, wherein the marker is in the test solution.

39. The system of claim 37, wherein the marker is in the carrier fluid.

40. The system of claim 35, further comprising an element for controlling the flow speed of the at least one test solution continuously flowing in the at least one channel in response to the average flow speed measured by said computer system.

41. The system of claim 34, wherein the fluorescent signal is measured at least once during each PCR cycle.

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42. The system of claim 34, wherein the detector is configured to detect a signal from amplification products and a signal from a marker at a plurality of locations along said defined section of said at least one channel.

43. The system of claim 42, wherein the detector is configured to independently detect signals from the amplified product and the marker.

44. The system of claim 34, wherein the at least one channel comprises a plurality of channels.

45. The system of claim 34, wherein the at least one test solution comprises a plurality of test solutions.

46. The system of claim 34, wherein the at least one carrier fluid comprises a plurality of carrier fluids.

47. The system of claim 34, further comprising a sensor to detect the temperature of the thermal transfer element and to provide feedback information for controlling the temperature cycles.

48. The system of claim 42, further comprising means for analyzing the amplified product.

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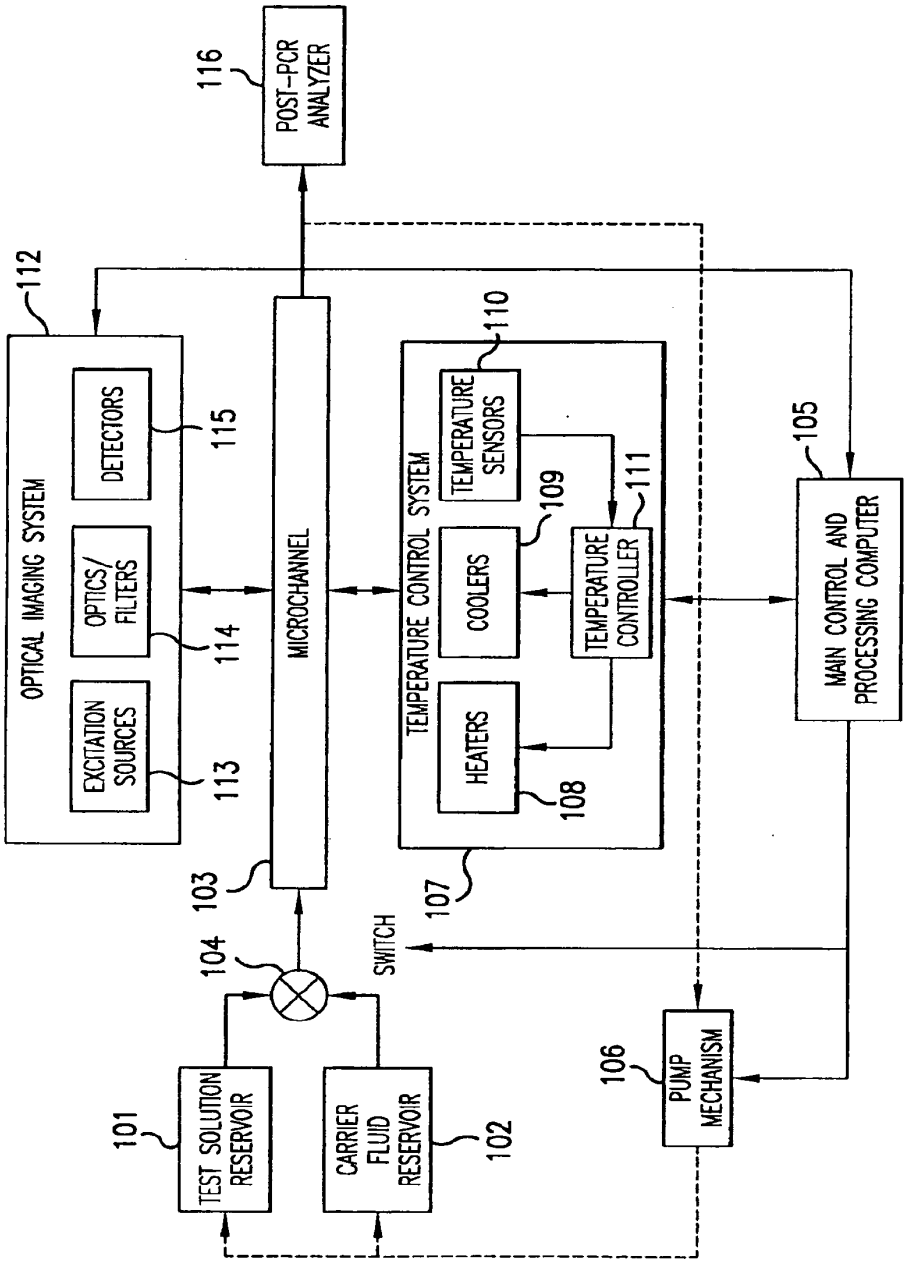

116
POST-PCR ANALYZER
112
OPTICAL IMAGING SYSTEM
DETECTORS
OPTICS/ FILTERS
EXCITATION SOURCES
115
114
113
MICROCHANNEL
103
110
TEMPERATURE CONTROL SYSTEM
TEMPERATURE SENSORS
COOLERS
HEATERS
TEMPERATURE CONTROLLER
109
111
108
107
105
MAIN CONTROL AND PROCESSING COMPUTER
SWITCH
104
101
TEST SOLUTION RESERVOIR
CARRIER FLUID RESERVOIR
102
106
PUMP MECHANISM

FIG. 1

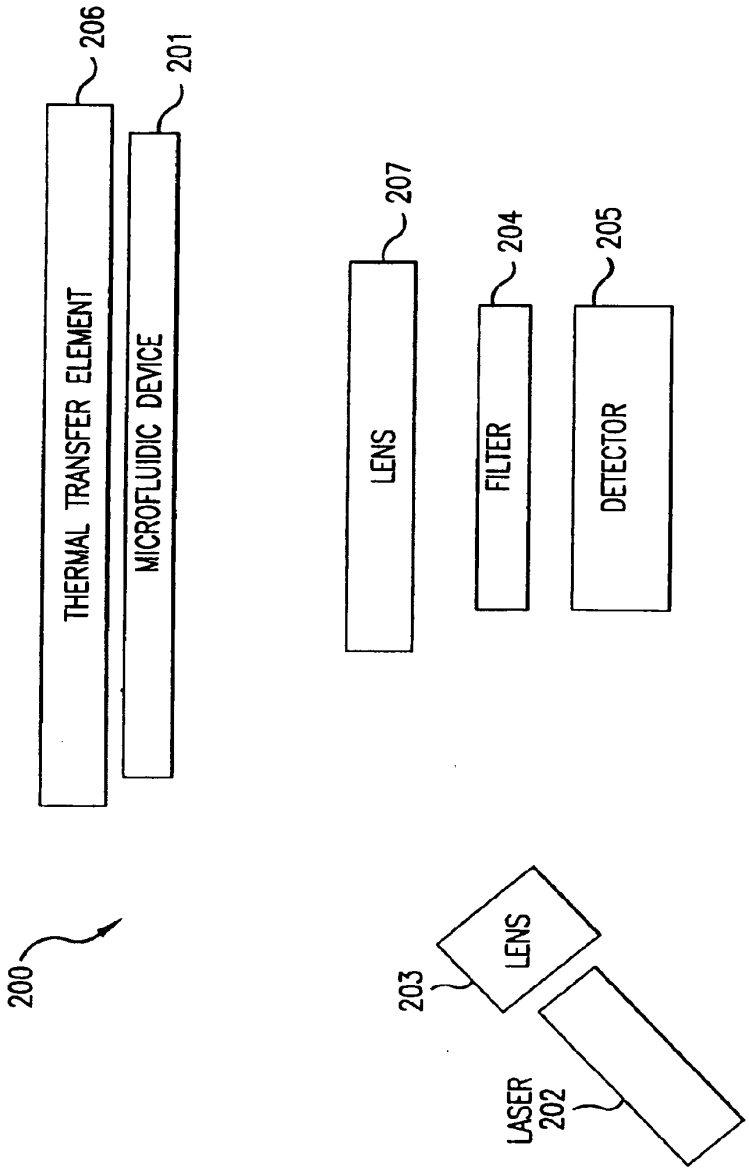

206
201
THERMAL TRANSFER ELEMENT
MICROFLUIDIC DEVICE
207
204
205
LENS
FILTER
DETECTOR
200
203
LENS
LASER
202

FIG.2

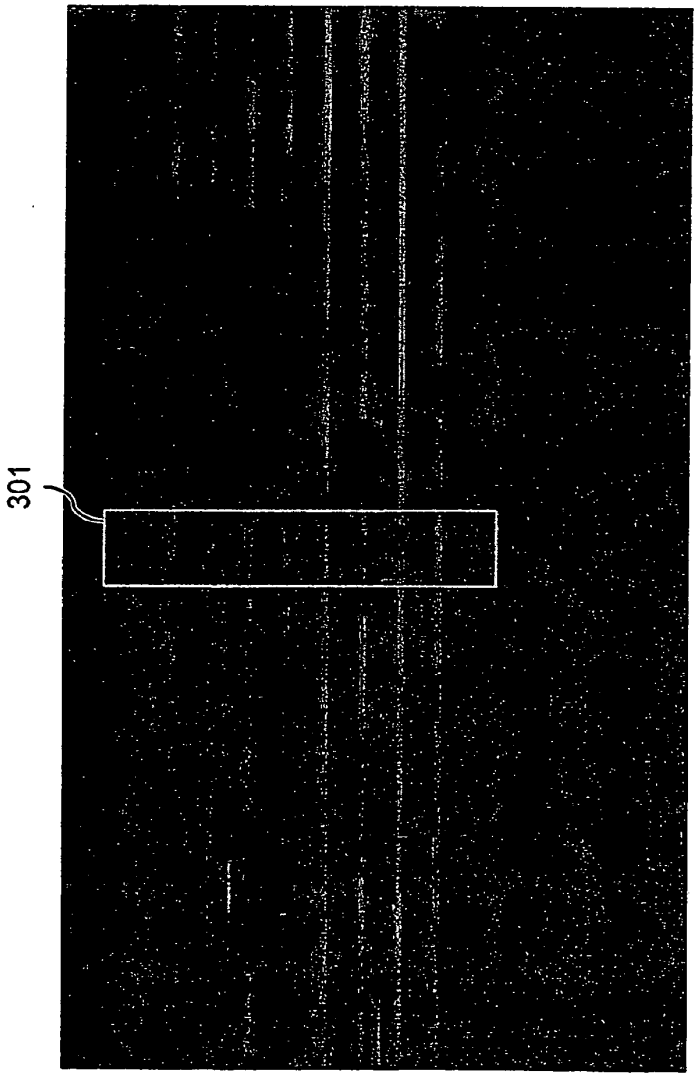


FIG.3A

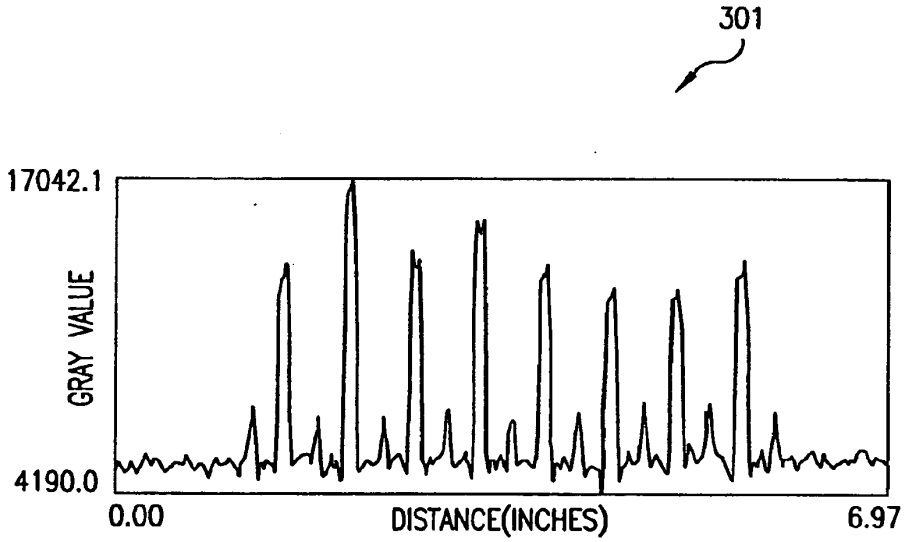


FIG.3B

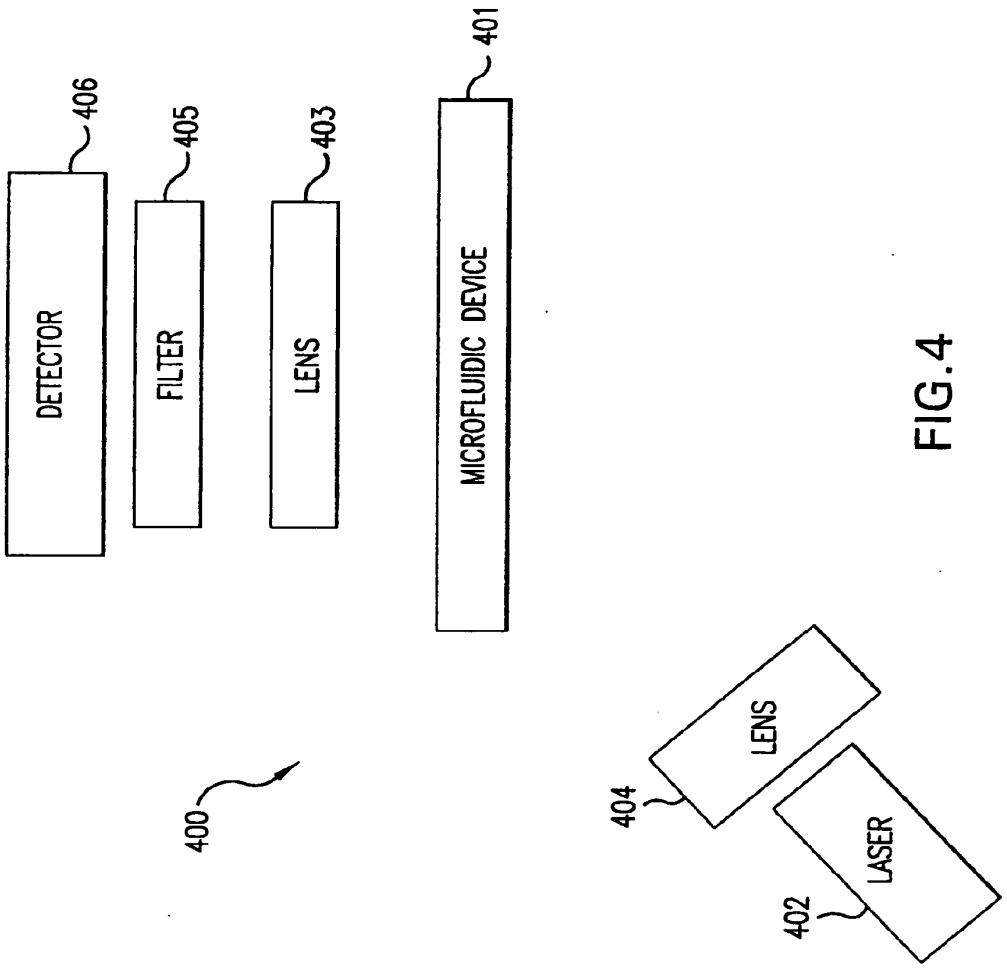

406
405
403
401
DETECTOR
FILTER
LENS
MICROFLUIDIC DEVICE
400
404
LENS
LASER
402

FIG.4

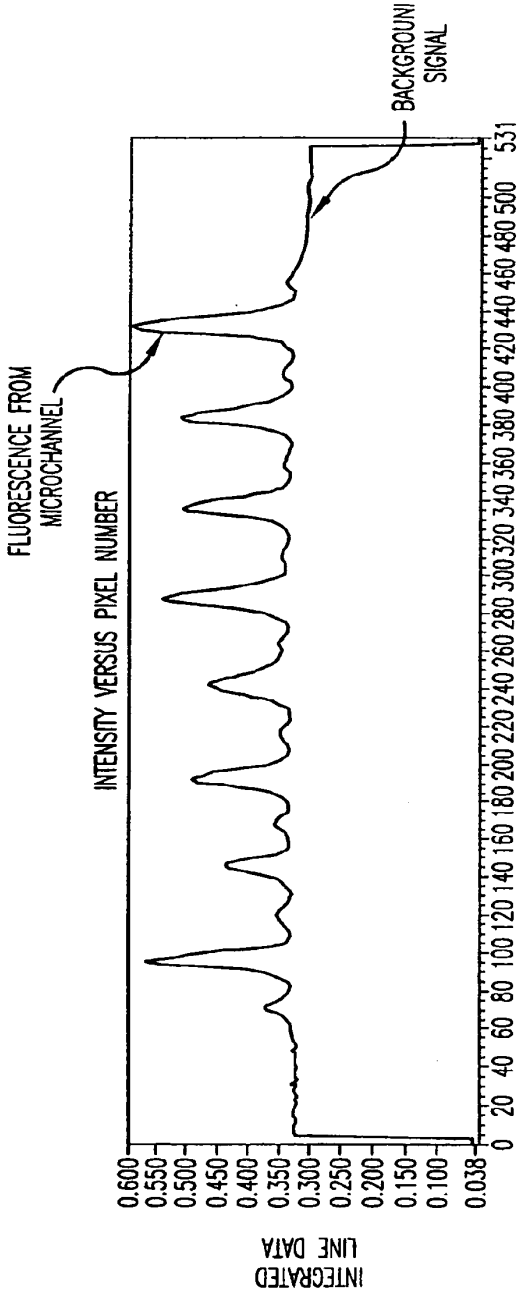

BACKGROUND SIGNAL
FLUORESCENCE FROM MICROCHANNEL
INTENSITY VERSUS PIXEL NUMBER
INTEGRATED LINE DATA
0.600
0.550
0.500
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.038
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460 480 500 531

FIG.5

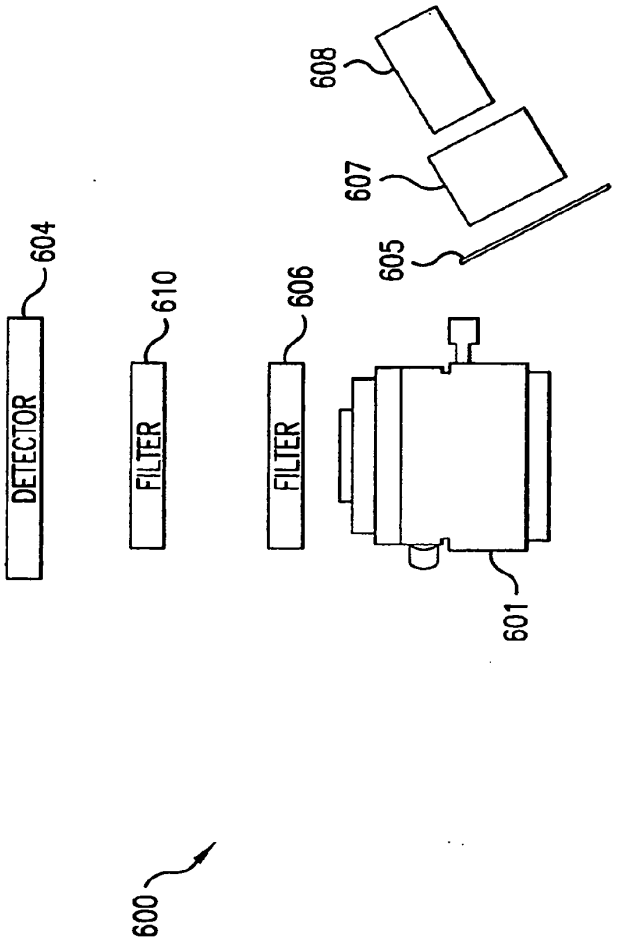


FIG.6