PTO/AIA/15 (10-17)
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UTILITY PATENT APPLICATION TRANSMITTAL

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

Attorney Docket No.	38075-716.302
First Named Inventor	Emil P. Kartalov
Title	**SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS**
Priority Mail Express® Label No.	e-Filed on March 7, 2018

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☐ **Fee Transmittal Form** (PTO/SB/17 or equivalent)
2. ☐ **Applicant asserts small entity status.** See 37 CFR 1.27
3. ☐ **Applicant certifies micro entity status.** See 37 CFR 1.29. Applicant must attach form PTO/SB/15A or B or equivalent.
4. ☒ **Specification** [*Total Pages* 70] Both the claims and abstract must start on a new page. *(See MPEP § 608.01(a) for information on the preferred arrangement)*
5. ☒ **Drawing(s)** (35 U.S.C. 113) [*Total Sheets* 8]
6. **Inventor's Oath or Declaration** [*Total Pages* 3] *(including substitute statements under 37 CFR 1.64 and assignments serving as an oath or declaration under 37 CFR 1.63(e))*
   a. ☐ Newly executed (original or copy)
   b. ☒ A copy from a prior application (37 CFR 1.63(d))
7. ☒ **Application Data Sheet** ** See note below.* See 37 CFR 1.76 (PTO/AIA/14 or equivalent)
8. **CD-ROM or CD-R** in duplicate, large table, or Computer Program *(Appendix)*
   ☐ Landscape Table on CD
9. **Nucleotide and/or Amino Acid Sequence Submission** *(if applicable, items a. - c are required)*
   a. ☐ Computer Readable Form (CRF)
   b. ☐ Specification Sequence Listing on:
      i. ☐ CD-ROM or CD-R (2 copies); or
      ii. ☐ Paper
   c. ☐ Statements verifying identity of above copies

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

ACCOMPANYING APPLICATION PAPERS

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

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

19. CORRESPONDENCE ADDRESS

☒ The address associated with Customer Number: 021971 **OR** ☐ Correspondence address below

Name					
Address					
City		State		Zip Code	
Country		Telephone		Email	

Signature	/Kyle Bruner/	Date	March 7, 2018
Name (Print/Type)	Kyle Bruner	Registration No. (Attorney/Agent)	75,716

This collection of information is required by 37 CFR 1.53(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313 - 1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313 - 1450.**

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

First Named Inventor:	Emil P. Kartalov	Nonprovisional Application Number (if known):	38075-716.302
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS		

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

1. The processing fee set forth in 37 CFR 1.17(i), the prioritized examination fee set forth in 37 CFR 1.17(c), and if not already paid, the publication fee set forth in 37 CFR 1.18(d) have been filed with the request. The basic filing fee, search fee, examination fee, and any required excess claims and application size fees are filed with the request or have been already been paid.

2. The application contains or is amended to contain no more than four independent claims and no more than thirty total claims, and no multiple dependent claims.

3. The applicable box is checked below:

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

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

---OR---

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

ii. An executed oath or declaration under 37 CFR 1.63 is filed with the application.

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

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

Signature /Kyle Bruner/	Date March 7, 2018
Name (Print/Typed) Kyle Bruner	Practitioner Registration Number 75,716

Note: *Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required in accordance with 37 CFR 1.33 and 11.18. Please see 37 CFR 1.4(d) for the form of the signature. If necessary, submit multiple forms for more than one signature, see below*.*

☒ *Total of 1 forms are submitted.

9760152_1.doc

PTO/SB/413C (05-11)
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REQUEST FOR FIRST ACTION INTERVIEW (FULL PILOT PROGRAM)

Attorney Docket Number: 38075-716.302	Application Number (if known): Herewith	Filing date: March 7, 2018
First Named Inventor: Emil P. Kartalov	Title: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

APPLICANT HEREBY REQUESTS A FIRST ACTION INTERVIEW IN THE ABOVE-IDENTIFIED APPLICATION. See Instruction Sheet on page 2.

1. The application must contain three (3) or fewer independent claims and twenty (20) or fewer total claims.

2. The application must not contain any multiple dependent claims.

3. By filing this request:

   Applicant is agreeing to make an election without traverse if the Office determines that the claims are not obviously directed to a single invention; and

   Applicant is agreeing not to request for a refund of the search fee and any excess claims fee paid in the application after the mailing or notification of the pre-interview communication prepared by the examiner.

4. Other attachments: _____

Signature /Kyle Bruner/	Date March 7, 2018
Name (Print/Typed) Kyle Bruner	Registration Number 75,716

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

☒ *Total of 1 forms are submitted.

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REQUEST FOR FIRST ACTION INTERVIEW (FULL PILOT PROGRAM)

Attorney Docket Number: 38075-716.302	Application Number (if known): Herewith	Filing date: March 7, 2018
First Named Inventor: Emil P. Kartalov	Title: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

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4. Other attachments: _____

Signature /Kyle Bruner/	Date March 7, 2018
Name (Print/Typed) Kyle Bruner	Registration Number 75,716

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

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

Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

As the below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number 13/756,760

filed on February 1, 2013.

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

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

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

WARNING:

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

LEGAL NAME OF INVENTOR

Inventor: Emil Kartalov Date (Optional): _____

Signature: Emil P. Kartalov

Note: An application data sheet (PTO/AIA/14 or equivalent), including naming the entire inventive entity, must accompany this form. Use an additional PTO/SB/AIA01 form for each additional inventor.

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

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LEGAL NAME OF INVENTOR

Inventor: Aditya Rajagopal Date (Optional): 02/28/2013

Signature: _____

Note: An application data sheet (PTO/AIA/14 or equivalent), including naming the entire inventive entity, must accompany this form. Use an additional PTO/SB/AIA01 form for each additional inventor.

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LEGAL NAME OF INVENTOR

Inventor: Axel Scherer Date (Optional): 2/28/13

Signature: Axel Scherer

Note: An application data sheet (PTO/AIA/14 or equivalent), including naming the entire inventive entity, must accompany this form. Use an additional PTO/SB/AIA01 form for each additional inventor.

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WSGR Docket No. 38075-716.302

PATENT APPLICATION

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Inventor(s): Emil P. Kartalov
Citizen of USA, Residing at
Pasadena, California

Aditya Rajagopal
Citizen of USA, Residing at
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Axel Scherer
Citizen of Germany, Residing at
Barnard, Vermont

Assignee: California Institute of Technology
1200 East California Boulevard
Pasadena, CA 91125

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Electronically Filed on March 7, 2018

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SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

CROSS-REFERENCE

[0001] This application is a continuation of U.S. Patent Application No. 14/451,876, filed August 5, 2014, which is a continuation of U.S. Patent Application No. 13/756,760, filed February 1, 2013, which claims the benefit of U.S. provisional applications 61/594,480, filed February 3, 2012 and 61/703,093, filed September 19, 2012. The content of these applications is incorporated herein by reference in its entirety.

BACKGROUND

[0002] 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 sequencing). There is a need for multiplexed reactions that can be carried out in a single solution.

SUMMARY OF THE INVENTION

[0003] 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.

[0004] 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.

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California Institute of Technology - Ex. 2003 Page 9

[0005] 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).

[0006] 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.

[0007] 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.

[0008] 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.

[0009] 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.

[0010] 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.

[0011] In some cases, an assay provided herein is performed with reagents that are lyophilized prior to use.

[0012] 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.

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California Institute of Technology - Ex. 2003 Page 10

[0013] 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.

[0014] In some examples, at least one cumulative measurement is performed on a solution.

[0015] 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 wavelength, a chemiluminescence intensity, a fluorescence bleaching rate, a chemiluminescence bleaching rate, and combinations thereof.

[0016] 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.

[0017] 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.

[0018] 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.

[0019] In some examples, information concerning the presence or absence of an analyte is transmitted through a computer network.

[0020] 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.

[0021] 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

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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.

[0022] In some cases, at least one of the analytes is a positive control analyte.

[0023] 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.

[0024] 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.

[0025] In certain examples, an assay provided herein is a liquid phase assay.

[0026] In some cases, an assay provided herein is quantitative.

[0027] 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.

[0028] 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.

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[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 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 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.

[0030] 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.

[0031] 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

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absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis.

[0032] 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.

[0033] 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.

[0034] 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 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

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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.

[0035] 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.

[0036] 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.

[0037] 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

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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.

[0038] 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.

[0039] 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.

[0040] 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.

[0041] 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.

[0042] 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

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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

[0043] 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

[0044] 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:

[0045] 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.

[0046] 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.

[0047] Fig. 3 shows chromatograms of experimental results obtained as described in Example 2.

[0048] 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.

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[0049] **Fig. 5** shows a schematic of two different methods of detecting wavelength and intensity of a light signal, such as a fluorescence emission signal.

[0050] **Fig. 6** shows a schematic of exemplary kits.

[0051] **Fig.** 7 shows chromatograms of experimental results obtained as described in Example 3.

[0052] **Fig. 8** shows a schematic of a legitimate result (top) and an illegitimate result (bottom).

DETAILED DESCRIPTION OF THE INVENTION

[0053] 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.

[0054] 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.

[0055] 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

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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.

[0056] 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.

[0057] 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.

[0058] 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).

[0059] 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

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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

[0060] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

[0061] 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".

[0062] 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.

[0063] 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).

[0064] 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.

[0065] 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.

[0066] 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.

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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.

[0067] 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.

[0068] 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.

[0069] 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.

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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

[0070] 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.

[0071] 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.

[0072] 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

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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

[0073] 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.

[0074] 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.

[0075] 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

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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.

[0076] 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

[0077] 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.

[0078] 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).

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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

[0079] 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.

[0080] 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

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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
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

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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
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

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[0081] 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.

[0082] 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.

[0083] 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

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[0084] 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.

[0085] 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.

[0086] 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.

[0087] **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

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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
Mycobacterium	1	1	0	1
Dengue Virus	1	1	1	1

[0088] 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).

[0089] 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

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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

[0090] 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.

[0091] 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.

[0092] 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.

[0093] 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

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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.

[0094] 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.

[0095] 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.

[0096] 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

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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.

[0097] 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 $M = (P * T) + 1$.

[0098] 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	
	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	

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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***	

[0099] 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

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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.

[00100] 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.
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

[00101] 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,

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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.

[00102] 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
	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

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		15	***15***	***15***	***15***

[00103] 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 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.

[00104] 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

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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.

[00105] 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

[00106] 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.

[00107] 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.

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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.

[00108] 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*.

[00109] 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.

[00110] 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

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(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.

[00111] 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.*

[00112] 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,*

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Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, Rhinosporidium seeberi, Sarcocystis bovihominis, Sarcocystis suihominis, Schistosoma mansoni, Toxoplasma gondii, Trichomonas vaginalis, Trypanosoma brucei, and *Trypansoma cruzi.*

[00113] 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.*

[00114] 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.

[00115] 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 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.

[00116] 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.

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[00117] 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.

[00118] 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.

[00119] 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,

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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

[00120] 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.

[00121] 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.

[00122] 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.

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[00123] 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 laboratory instrumentation, including quantitative PCR machines. Intensity may be measured with a photodetector. A range of wavelengths may be referred to as a "channel."

[00124] 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.

[00125] 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).

[00126] 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

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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.

[00127] 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.

[00128] 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.

[00129] 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

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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.

[00130] 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.

[00131] 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, 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.

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[00132] 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).

[00133] 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).

[00134] 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.

[00135] 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,

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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, or 50 second components of a signal.

[00136] 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

[00137] 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).

[00138] 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,

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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.

[00139] 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.

[00140] 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.

[00141] 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

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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.

[00142] 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.

[00143] 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.

[00144] 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.

[00145] 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,

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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.

[00146] 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.

[00147] 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.

[00148] 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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[00149] 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.

[00150] 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.

[00151] 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.

[00152] 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

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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

[00153] 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.

[00154] **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.**

[00155] 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.**

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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 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.**

[00156] 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.

[00157] 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.

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[00158] 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.

[00159] 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.

[00160] 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.

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VI. Compositions and Kits

[00161] 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.

[00162] 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.

[00163] 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.

[00164] 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.

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[00165] **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.**

[00166] **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

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[00167] 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.

[00168] 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.

[00169] 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

[00170] 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

[00171] 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

[00172] 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).

[00173] 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

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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.

[00174] 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 sequence (3' to 5')	CAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATA 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

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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
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

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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 (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/
Probe 4 (3' to 5')	/**5Cy5**/GGATATCCAATGGTACAGGCAACATCGT/3IAbRQSp/

3. Polymerase Chain Reactions

[00175] 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).

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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

[00176] 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

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	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
	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

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

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

[00177] 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.

[00178] 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

[00179] 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.

[00180] 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

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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.

[00181] 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.

[00182] 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.

[00183] **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.

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

[00184] 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

[00185] 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

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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 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
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

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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**

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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 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

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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

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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

[00186] 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.

[00187] **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.

[00188] 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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CLAIMS

WHAT IS CLAIMED IS:

1. A method of unambiguously detecting any unique combination of presence or absence of at least five polynucleotide analytes in a plurality of droplets, the method comprising:
   (a) providing a sample comprising, or potentially comprising, at least one of said at least five polynucleotide analytes;
   (b) forming a mixture of said sample and at least five hybridization probes, each comprising one or more fluorophores selected from at most four fluorophores;
   (c) partitioning said mixture into said plurality of droplets;
   (d) exciting said fluorophores to generate one or more signals if one or more of said at least five polynucleotide analytes is present in said plurality of droplets, wherein said one or more signals comprise at least one signal generated by excitement of said one or more fluorophores; and
   (e) measuring said one or more signals to generate a cumulative measurement, wherein said cumulative measurement corresponds to the presence of a unique combination of presence or absence of said at least five polynucleotide analytes in said sample, thereby detecting the presence or absence of said at least five polynucleotide analytes, in any unique combination of presence or absence;
   wherein the method does not require any step of immobilization of said at least five polynucleotide analytes or mass spectrometry.
2. The method of claim 1, wherein each of said at least five hybridization probes is complementary to one of said at least five polynucleotide analytes.
3. The method of claim 1, wherein said at least five hybridization probes comprise one or more fluorophores selected from one, two, three, or four fluorophores.
4. The method of claim 1, wherein, in (b), a droplet of said plurality of droplets comprises said at least five hybridization probes.
5. The method of claim 1, wherein, in (b), a droplet of said plurality of droplets comprises one or more of said at least five polynucleotide analytes.
6. The method of claim 1, wherein, in (b), a droplet of said plurality of droplets comprises at most one of said at least five polynucleotide analytes.

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7. The method of claim 1, wherein said one or more signals is generated during a polymerase chain reaction.
8. The method of claim 1, wherein said at least five polynucleotide analytes are at least seven polynucleotide analytes.
9. The method of claim 1, wherein said at least five hybridization probes are at least seven hybridization probes.
10. The method of claim 1, wherein said one or more polynucleotide analytes comprise cell-free nucleic acid, tumor nucleic acid, fetal nucleic acid, viral nucleic acid, or any combination thereof.
11. The method of claim 1, wherein said sample is a biological sample derived from a subject.
12. The method of claim 11, wherein detecting the presence or absence of said at least five polynucleotide analytes indicates the presence of cancer or an infection with a pathogen in said subject.
13. The method of claim 1, further comprising transmitting information concerning the presence or absence of said at least five polynucleotide analytes through a computer network.
14. The method of claim 1, further comprising providing information concerning the presence or absence of said at least five polynucleotide analytes to a health care professional, wherein the health care professional makes a clinical decision based on said information.
15. The method of claim 14, wherein said clinical decision is a treatment decision.
16. The method of claim 15, wherein said treatment comprises treatment with an antibiotic or an anti-cancer therapeutic.
17. The method of claim 1, wherein at least part of said method is performed using a computer.
18. The method of claim 17, wherein said computer is located on a remote server.
19. The method of claim 17, wherein said computer is located on a droplet reader.
20. The method of claim 1, wherein each of said at least five hybridization probes are provided at a given concentration such that said cumulative measurement corresponds to said presence of said unique combination.

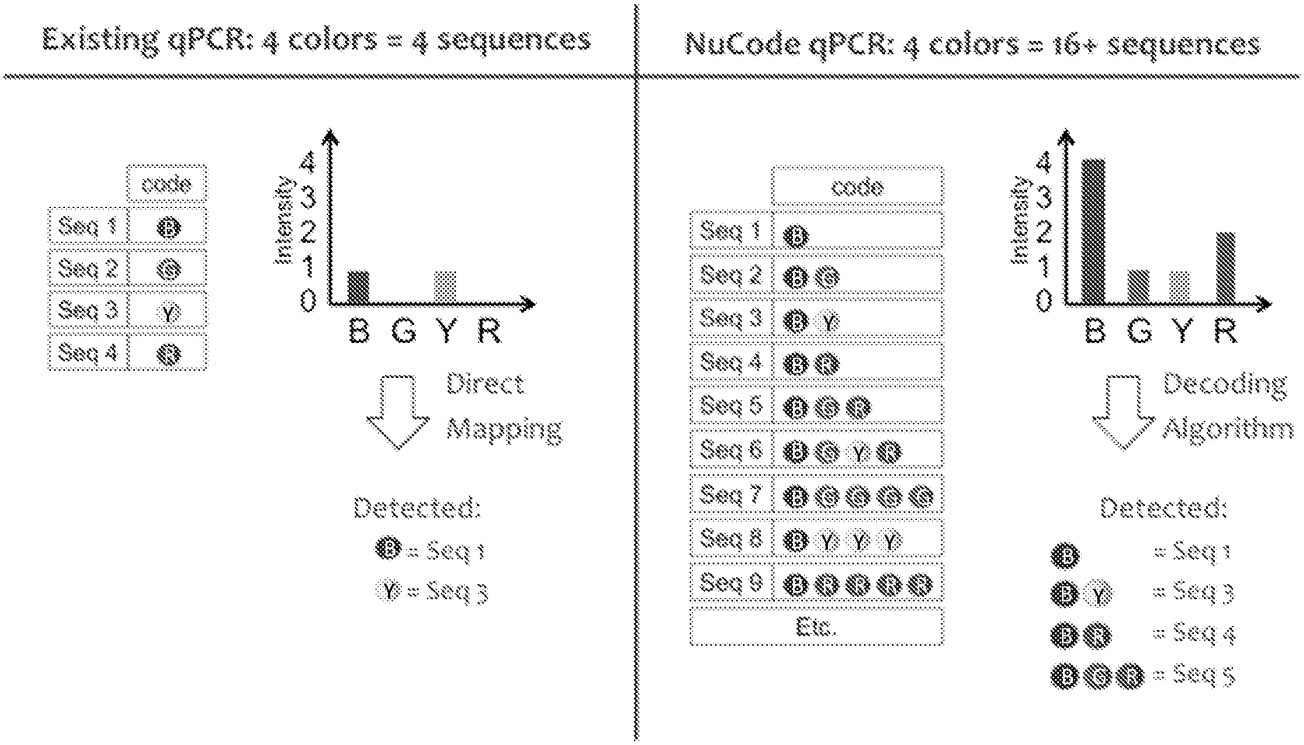
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ABSTRACT

This disclosure provides methods, systems, compositions, and kits for the multiplexed detection of a plurality of analytes in a sample. In some examples, this disclosure provides methods, systems, compositions, and kits wherein multiple analytes may be detected in a single sample volume by acquiring a cumulative measurement or measurements of at least one quantifiable component of a signal. In some cases, additional components of a signal, or additional signals (or components thereof) are also quantified. Each signal or component of a signal may be used to construct a coding scheme which can then be used to determine the presence or absence of any analyte.

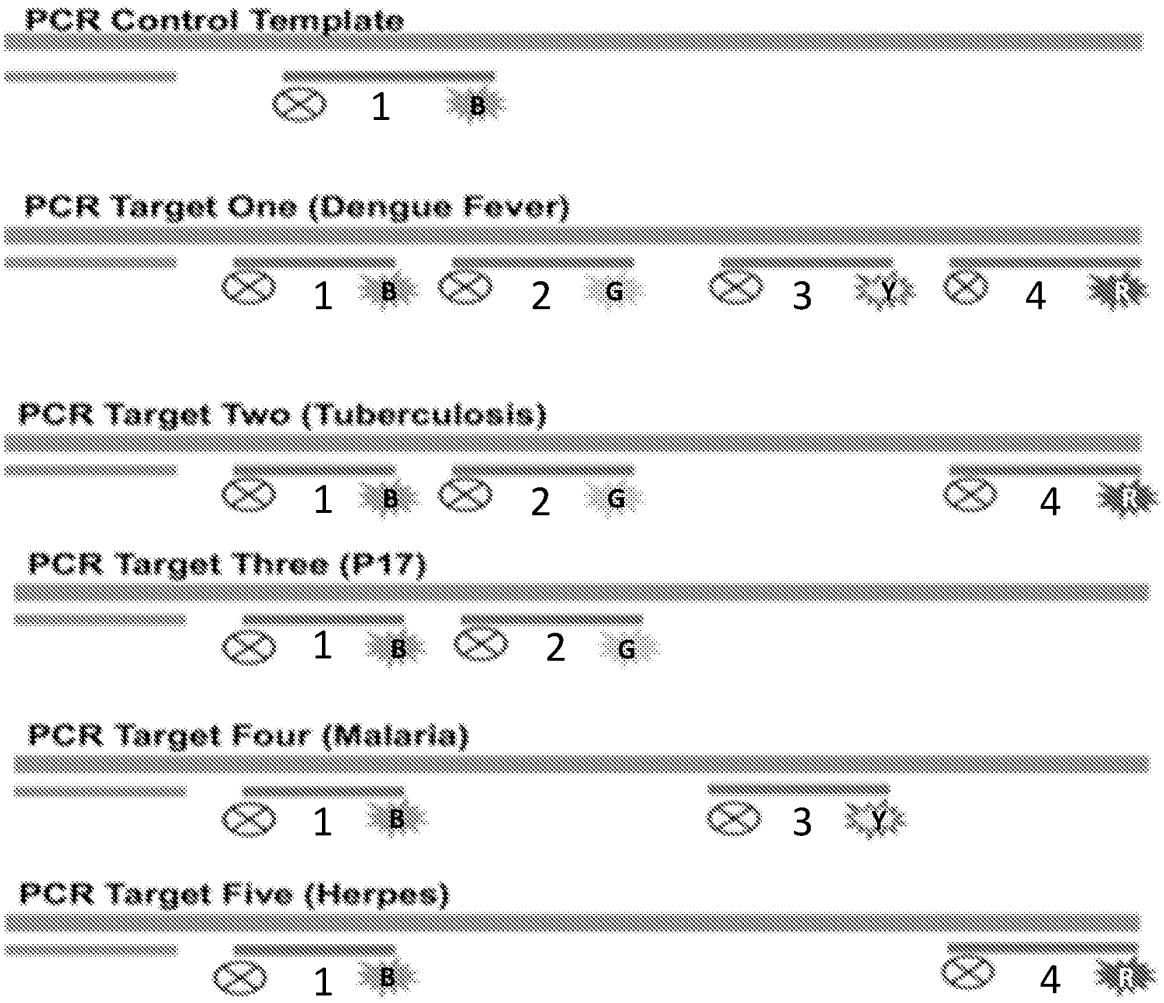
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Fig. 1



B: Blue
G: Green
Y: Yellow
R: Red

Fig. 2



B: Blue
G: Green
Y: Yellow
R: Red

Fig. 3

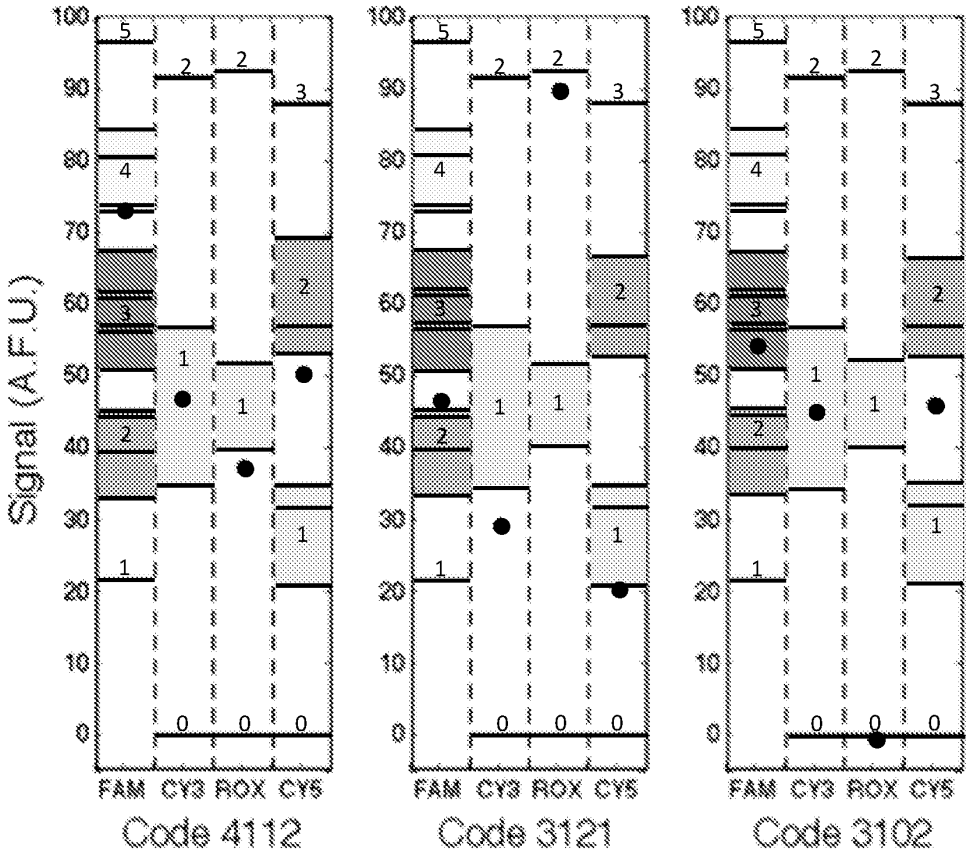


Fig. 4

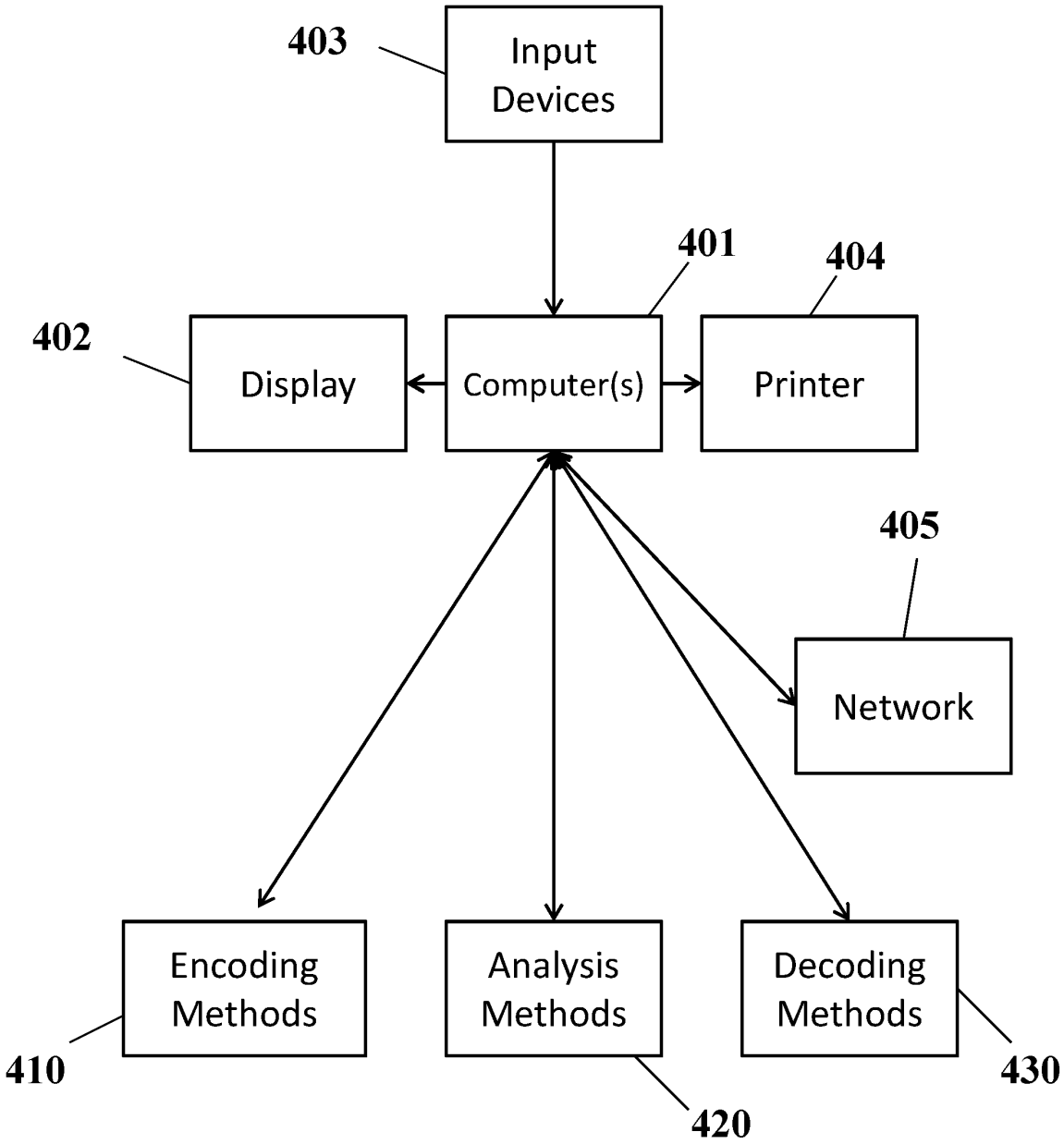

403
Input Devices
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404
402
Display
Computer(s)
Printer
405
Network
Encoding Methods
Analysis Methods
Decoding Methods
410
420
430

Fig. 5

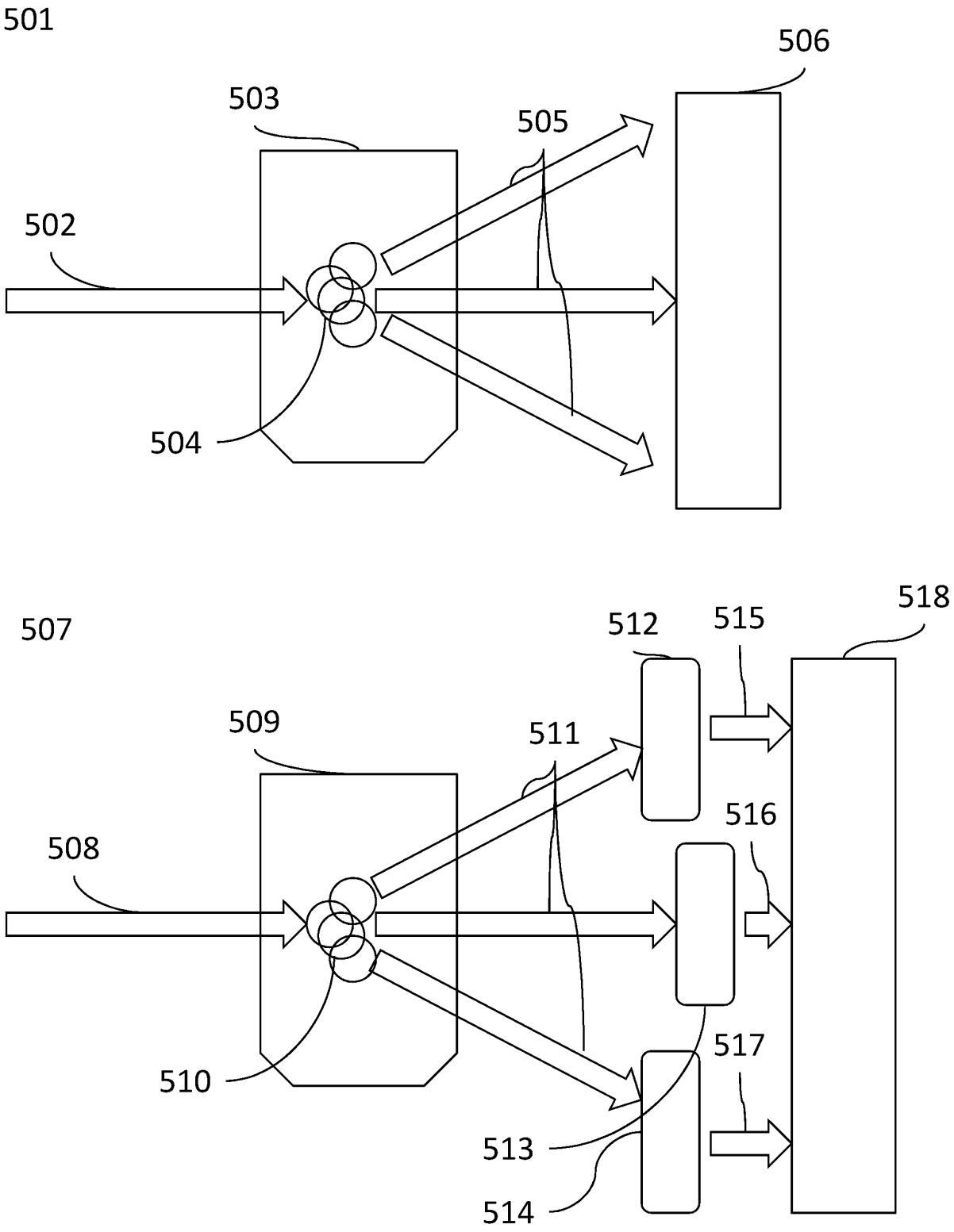

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Fig. 6

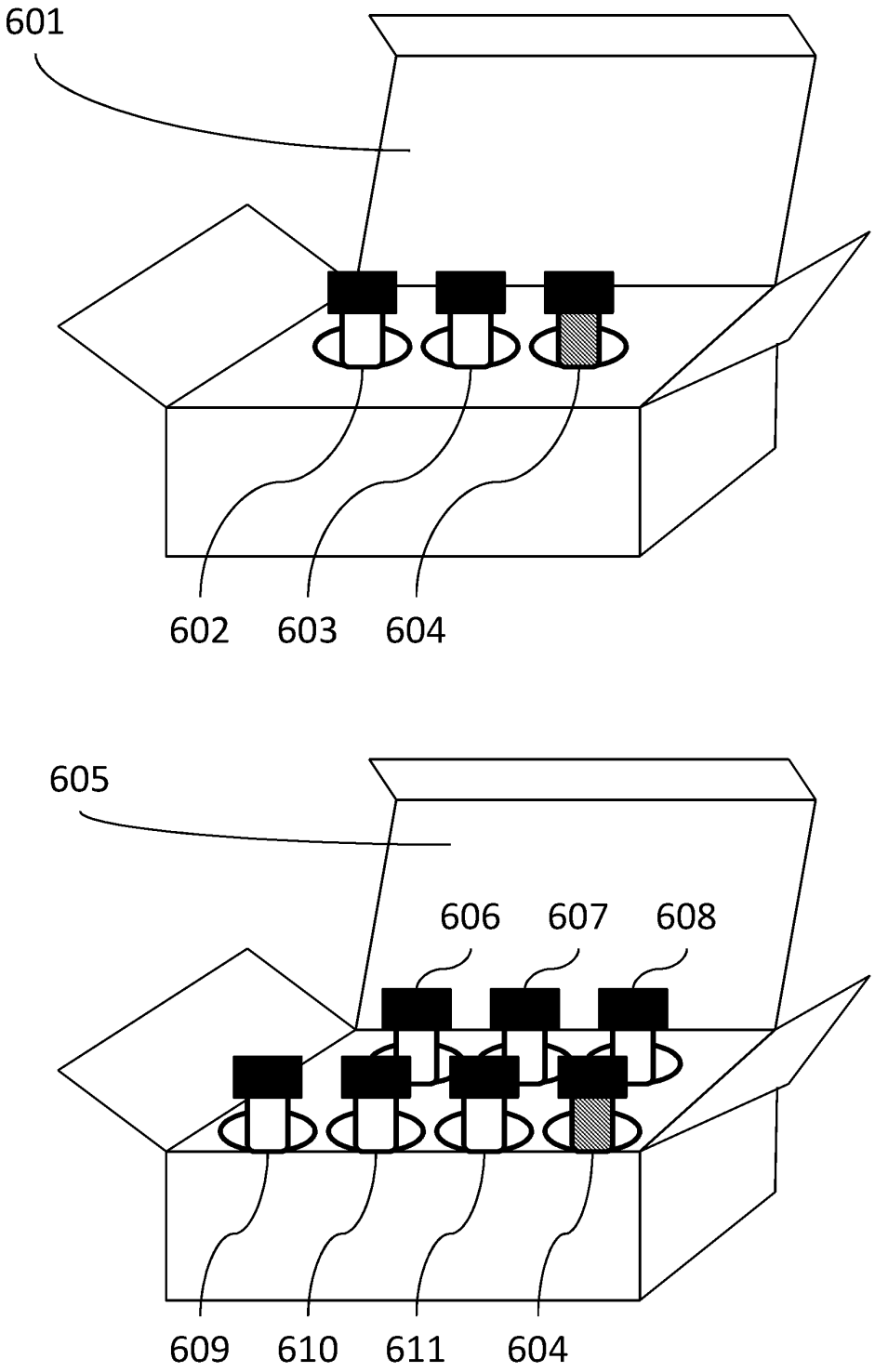


Fig. 7

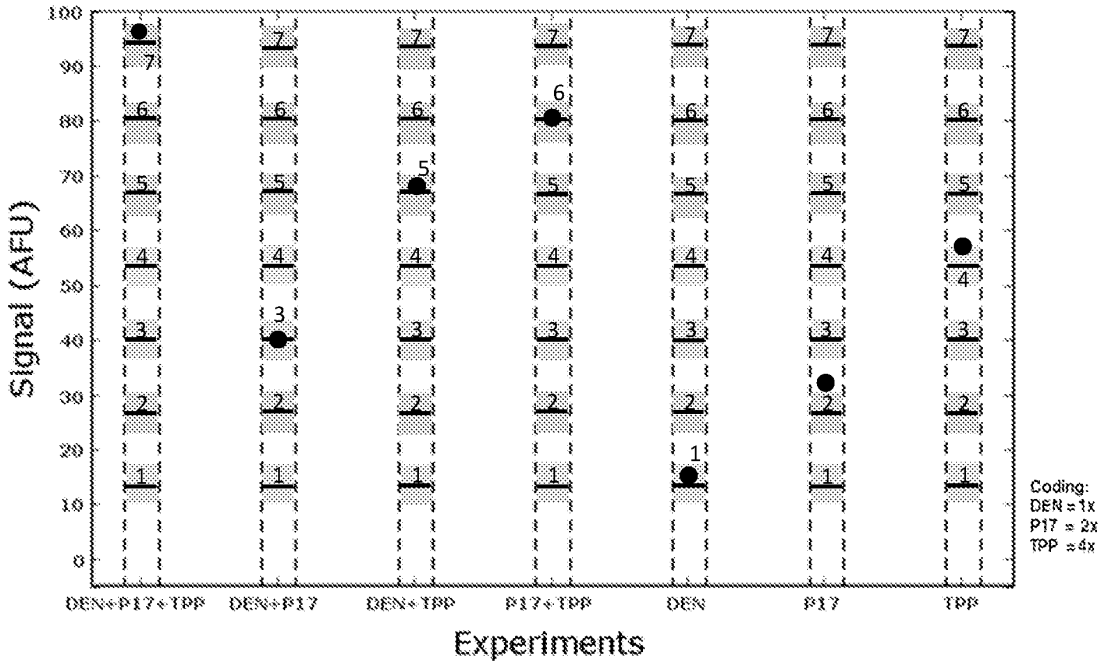
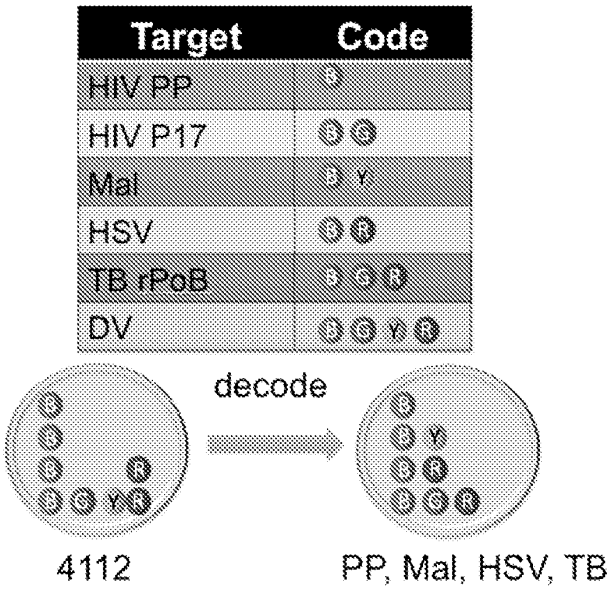
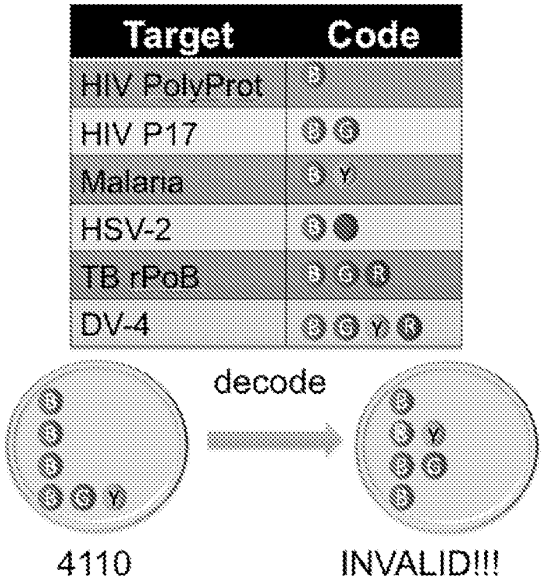

Signal (AFU)
Experiments
DEN+P17+TPP
DEN+P17
DEN+TPP
P17+TPP
DEN
P17
TPP
Coding:
DEN = 1x
P17 = 2x
TPP = 4x

Fig. 8


Target
Code
HIV PP
HIV P17
Mal
HSV
TB rPoB
DV
decode
4112
PP, Mal, HSV, TB


Target
Code
HIV PolyProt
HIV P17
Malaria
HSV-2
TB rPoB
DV-4
decode
4110
INVALID!!!

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil P. Kartalov
Filer:	J Kyle Bruner/Katherine Lopez
Attorney Docket Number:	38075-716.302

Filed as Large Entity

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

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
UTILITY APPLICATION FILING	1011	1	300	300
UTILITY SEARCH FEE	1111	1	660	660
UTILITY EXAMINATION FEE	1311	1	760	760
REQUEST FOR PRIORITIZED EXAMINATION	1817	1	4000	4000
Pages:				
Claims:				
Miscellaneous-Filing:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
PUBL. FEE- EARLY, VOLUNTARY, OR NORMAL	1504	1	0	0
PROCESSING FEE, EXCEPT PROV. APPLS.	1830	1	140	140
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**5860**

Electronic Acknowledgement Receipt	
EFS ID:	31984187
Application Number:	15914356
International Application Number:	
Confirmation Number:	2457
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil P. Kartalov
Customer Number:	21971
Filer:	J Kyle Bruner/Katherine Lopez
Filer Authorized By:	J Kyle Bruner
Attorney Docket Number:	38075-716.302
Receipt Date:	07-MAR-2018
Filing Date:	
Time Stamp:	14:44:38
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$5860
RAM confirmation Number	030818INTEFSW00000990232415
Deposit Account	232415
Authorized User	J Bruner
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.21 (Miscellaneous fees and charges)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Application Data Sheet	CIT_38075_716_302_Signed_ADS.pdf	3163613 05c1d76a6ef87695bba182d69ef4f46accda4915	no	9
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
2	Transmittal of New Application	CIT_38075_716_302_Signed_Trans.pdf	215085 6e6d11a9c8e3336b6e362e580d0e912031e72706	no	1
Warnings:					
Information:					
3	TrackOne Request	CIT_38075_716_302_Signed_Track_One_Request.pdf	159535 8cfa6bf593f7ffeac07dafeba1f5ff25929aece3	no	1
Warnings:					
Information:					
4	First Action Interview - Enrollment Request	CIT_38075_716_302_Signed_Request_First_Action.pdf	133224 20bfe4dc22ee380f04637726e76408e5e2eff2d0a	no	1
Warnings:					
Information:					
5	Oath or Declaration filed	CIT_38075_716_302_Signed_Decs.pdf	250843 c83078092d6e870f9b186a91c0fb94b59eeaf027	no	3
Warnings:					
Information:					
6		CIT_38075_716_302_Appl.pdf	509409 d5cf78840b9fc228aab75db9b3e01833e5fb8df8	yes	70

	Multipart Description/PDF files in .zip description				
	Document Description	**Start**	**End**		
	Specification	1	67		
	Claims	68	69		
	Abstract	70	70		
Warnings:					
Information:					
7	Drawings-only black and white line drawings	CIT_38075_716_302_Figs.pdf	1438376 8a3d5bcee52775f6bca9550d69a4f3297e46b584	no	8
Warnings:					
Information:					
8	Fee Worksheet (SB06)	fee-info.pdf	40405 4c934b35a57118b2b5d25c2b07dee055e61dd71e	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	5910490		

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

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

PTO/AIA/14 (11-15)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	38075-716.302
	Application Number	
Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

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

Secrecy Order 37 CFR 5.2:

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

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Emil	P.	Kartalov	

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

City	Pasadena	State/Province	CA	Country of Residence	US

Mailing Address of Inventor:

Address 1	2133 E. Mountain Street		
Address 2			
City	Pasadena	State/Province	CA
Postal Code	91104	Country	US

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Aditya		Rajagopal	

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

City	Orange	State/Province	CA	Country of Residence	US

Mailing Address of Inventor:

Address 1	221 W. Sparkleberry Avenue		
Address 2			
City	Orange	State/Province	CA
Postal Code	92865	Country	US

Inventor 3 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Axel		Scherer	

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

PTO/AIA/14 (11-15)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	38075-716.302
	Application Number	
Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

City	Pasadena	State/Province	CA	Country of Residence	US

Mailing Address of Inventor:

Address 1	1200 East California Boulevard		
Address 2			
City	Pasadena	State/Province	CA
Postal Code	91125	Country	US

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

Correspondence Information:

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

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

Customer Number	21971		
Email Address	patentdocket@wsgr.com	Add Email	Remove Email

Application Information:

Title of the Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS		
Attorney Docket Number	38075-716.302	Small Entity Status Claimed	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	8	Suggested Figure for Publication (if any)	

Filing By Reference:

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

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

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

Publication Information:

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

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

PTO/AIA/14 (11-15)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	38075-716.302
	Application Number	
Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

Representative Information:

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

Please Select One:	◉ Customer Number	○ US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	21971		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	Continuation of	14/451876	2014-08-05

Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
14/451876	Continuation of	13/756760	2013-02-01	8838394	2014-09-16

Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13/756760	Claims benefit of provisional	61/594480	2012-02-03

Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13/756760	Claims benefit of provisional	61/703093	2012-09-19

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

Foreign Priority Information:

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	38075-716.302
	Application Number	
Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

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

Remove

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

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

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

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

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	38075-716.302
	Application Number	
Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

Authorization or Opt-Out of Authorization to Permit Access:

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

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

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

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

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

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

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

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

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

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

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

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	38075-716.302
	Application Number	
Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

Applicant Information:

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

Applicant 1

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

Clear

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

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

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

Organization Name	California Institute of Technology		
Mailing Address Information For Applicant:			
Address 1	1200 East California Boulevard		
Address 2			
City	Pasadena	**State/Province**	CA
Country	US	Postal Code	91125
Phone Number		Fax Number	
Email Address			

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

Assignee Information including Non-Applicant Assignee Information:

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

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	38075-716.302
	Application Number	
Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

Assignee 1

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

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

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

Mailing Address Information For Assignee including Non-Applicant Assignee:

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

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

Signature:

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

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

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

Signature	/Kyle Bruner/			Date (YYYY-MM-DD)	2018-03-07
First Name	Kyle	Last Name	Bruner	Registration Number	75,716

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

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	38075-716.302
	Application Number	
Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

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

Privacy Act Statement

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

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

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

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

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

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

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

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

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

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

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

DocCode – SCORE

SCORE Placeholder Sheet for IFW Content

Application Number: 15914356 Document Date: 03/07/2018

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

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

- Drawing

At the time of document entry (noted above):

- USPTO employees may access SCORE content via eDAN using the Supplemental Content tab, or via the SCORE web page.
- External customers may access SCORE content via PAIR using the Supplemental Content tab.

Form Revision Date: August 26, 2013

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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 7

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	Unassigned
Attorney Docket Number	38075-716.302

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	001	US-7930106	04-19-2011	CARRICK; James M.	
	002	US-8039215	10-18-2011	HIGUCHI; Russell et al.	
	003	US-8426132	04-23-2013	LI; Bin et al.	
	004	US-8455184	06-04-2013	ATCHLEY; Daniel H. et al.	
	005	US-8492094	07-23-2013	DIMITROV; Krassen et al.	
	006	US-8519115	08-27-2013	WEBSTER; Philippa J. et al.	
	007	US-8614061	12-24-2013	BRABETZ; Werner et al.	
	008	US-8771955	07-08-2014	REED; Michael W. et al.	
	009	US-8838394	09-16-2014	KARTALOV; Emil et al.	
	010	US-8877464	11-04-2014	BABIEL; Reiner et al.	
	011	US-8962250	02-24-2015	STANLEY; Keith	
	012	US-9133506	09-15-2015	KATZIR; Nir et al.	
	013	US-9222128	12-29-2015	SAXONOV; Serge et al.	
	014	US-9260761	02-16-2016	TYAGI; Sanjay et al.	
	015	US-9422593	08-23-2016	ROTHMANN; Thomas et al.	
	016	US-9447457	09-20-2016	CHUN; Jong Yoon et al.	
	017	US-20020146734	10-10-2002	ORTYN; William E. et al.	
	018	US-20070072211	03-29-2007	NEWTON; Nick et al.	
	019	US-20080124705	05-29-2008	KRAMER; Fred R.	
	020	US-20100151443	06-17-2010	XIANG; Qing et al.	
	021	US-20100233686	09-16-2010	HIGUCHI; Russell et al.	
	022	US-20110151550	06-23-2011	SAGNER; Gregor et al.	

Examiner Signature		Date Considered	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 2 of 7

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	Unassigned
Attorney Docket Number	38075-716.302

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	023	US-20130017971	01-17-2013	GEISS; Gary K. et al.	
	024	US-20130040841	02-14-2013	SAXONOV; Serge et al.	
	025	US-20130261019	10-03-2013	LIN; Chenxiang et al.	
	026	US-20140038195	02-06-2014	MALIK; Imran R. et al.	
	027	US-20140171341	06-19-2014	JOUVENOT; Yann et al.	
	028	US-20150057178	02-26-2015	KARTALOV; Emil et al.	
	029	US-20150140554	05-21-2015	SNYDER; Richard O. et al.	
	030	US-20170362636	12-21-2017	RAJAGOPAL; Aditya et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	001	CN-101570782-A	11-04-2009	CHUNHUA YANG [CN]	English Abstract	☒
	002	EP-1629108-B1	12-31-2014	PHRI PROPERTIES INC [US]		☐
	003	WO-03002979-A2	01-09-2003	ILLUMINA INC [US], et al.		☐
	004	WO-2004087950-A2	10-14-2004	ROCHE DIAGNOSTICS GMBH [DE], et al.		☐

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Sheet 3 of 7

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	Unassigned
Attorney Docket Number	38075-716.302

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	005	WO-2010128206-A1	11-11-2010	EXPRESSION ANALYTICS OY [FI], et al.		☐
	006	WO-2013096851-A1	06-27-2013	HARVARD COLLEGE [US]		☐
	007	WO-2013116780-A1	08-08-2013	CALIFORNIA INST OF TECHN [US], et al.		☐
	008	WO-2017173035-A1	10-05-2017	CHROMACODE INC [US]		☐

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Sheet 4 of 7

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	Unassigned
Attorney Docket Number	38075-716.302

NON-PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No.[1]	Include name of the author(in CAPITAL LETTERS),title of the article(when appropriate), title of the item (book,magazine,journal,serial,symposium,catalog,etc.),date,page(s),volume-issue number(s),publisher, city and/or country where published.	T[2]
	001	CHAPIN et al., Rapid microRNA profiling on encoded gel microparticles. Angewandte Chemie International Edition, 50(10):2289-2293, 2011.	☐
	002	European Patent Application No. 13744261.2 Extended European Search Report dated May 3, 2016.	☐
	003	GANDELMAN, et al., Novel Bioluminescent Quantitative Detection of Nucleic Acid Amplification in Real-Time, PLoS One, 2010, 5(11):e14155, 14 pages.	☐
	004	HAN et al., Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. Nature Biotechnology, 19(7):631-635, 2001.	☐
	005	Holland, et al., Detection of specific polymerase chain reaction product by utilizing the 5´ to 3´ exonuclease activity of Thermus aquaticus DNA polymerase. PNAS (USA) 88:7276–7280, 1991.	☐
	006	International Search Report and Written Opinin dated 11/16/2017 for International PCT Patent Application No. PCT/US2017/37682.	☐
	007	International search report and written report dated 06/22/2017 for PCT Application No. PCT/US2017/24933.	☐
	008	KLOSTRANEC et al., Convergence of quantum dot barcodes with microfluidics and signal processing for multiplexed high-throughout infectious disease diagnostics. Nano Letters, 7(9):2812-2818, 2007.	☐
	009	LI et al., Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes. Nature Biotechnology, 23(7):885-889, 2005.	☐

Examiner Signature		Date Considered	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 5 of 7

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	Unassigned
Attorney Docket Number	38075-716.302

NON-PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No.[1]	Include name of the author(in CAPITAL LETTERS),title of the article(when appropriate), title of the item (book,magazine,journal,serial,symposium,catalog,etc.),date,page(s),volume-issue number(s),publisher, city and/or country where published.	T[2]
	010	LIN et al., Self-assembled combinatorial encoding nanoarrays for multiplexed biosensing. Nano Letters, 7(2):507-512, 2007.	☐
	011	Notice of allowance dated 06/02/2014 for US Application No. 13/756,760.	☐
	012	Office action dated 01/24/2014 for US Application No. 13/756,760.	☐
	013	Office action dated 06/06/2017 for US Application No. 14/451,876.	☐
	014	Office action dated 11/23/2016 for US Application No. 14/451,876.	☐
	015	SAMBROOK, et al. Molecular Cloning: A Laboratory Manual. 2nd Edition, 1989.	☐
	016	U.S. Serial No. 15/623,974 Office Action dated February 22, 2018.	☐
	017	U.S. Serial No. 15/677,772 Office Action dated February 08, 2018.	☐
	018	XU et al., Multiplexed SNP genotyping using the Qbead system: a quantum dot-encoded microspere-based assay. Nucleic Acids Research, 31(8):e43, 2003.	☐

Examiner Signature		Date Considered	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 6 of 7

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	Unassigned
Attorney Docket Number	38075-716.302

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.

☐ 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	/Kyle Bruner/	Date (YYYY-MM-DD)	2018-03-22
Name/Print	Kyle Bruner	Registration Number	75,716

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

Electronic Acknowledgement Receipt

EFS ID:	32135095
Application Number:	15914356
International Application Number:	
Confirmation Number:	2457
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil P. Kartalov
Customer Number:	21971
Filer:	J Kyle Bruner/Judy Wan
Filer Authorized By:	J Kyle Bruner
Attorney Docket Number:	38075-716.302
Receipt Date:	22-MAR-2018
Filing Date:	
Time Stamp:	20:26:48
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Foreign Reference	CN101570782A.PDF	954701 e69d4f7bc607214dd6fa39ddfa6f6bb9043380e2	no	23

Warnings:

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2	Foreign Reference	EP1629108B1.PDF	7339191 65d612d71f0810d657f808278394ab68f7b17de8	no	26
Warnings:					
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4	Foreign Reference	WO13096851.PDF	1380354 a806d492e7a50c63c580ccab6de2c543a8c9a7cd	no	128
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5	Foreign Reference	WO2004087950A2.PDF	2588693 97613b05e6a6fca530ad3abdc77b4a91af06c30c	no	62
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6	Foreign Reference	WO2010128206A1.PDF	1535764 0a9926e2725d0b1ad2976e90e8c688b4c93718ff	no	42
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8	Foreign Reference	WO2017173035A1.PDF	2525583 5d8df6d2adf9a5ea0672cf50f162771e50c622d9	no	46
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Information:					
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17	Non Patent Literature	GANDELMAN-NOVEL-BIOLUMINESCENT-2010.pdf	840908 c62121b6479dc5b53b7bcf548c1736075c7533fb	no	14
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Warnings:					
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25	Non Patent Literature	XU-Multiplexed-SNP-2003.PDF	565915 a8e2f00652357ac17d321c7cf4c587618b55f4ce	no	6
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26	Non Patent Literature	EESR-05-03-16-EP137442612.PDF	999929 0ac2cecd5a93467b0ad126e9cdc1fc2e697edbac	no	20
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27		IDS-38075-716-302-03-22-18-2.pdf	327329 4171bf642bf6c76699b1f1ea650ca193b8cd33c5	yes	11

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Espacenet

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Detection kit and detection method for 8 species of pathogenic bacteria in dairy products

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Abstract of CN101570782 (A)

The invention discloses a detection kit and a detection method for 8 species of pathogenic bacteria in dairy products, which are used for food microbial contamination monitoring and detection. The kit comprises a detection solution A and a detection solution B, wherein the detection solution A and the detection solution B both contain 10 millimols of Tris.HCl, 50 millimols of KCl, 25 millimols of MgCl2, 2.5 millimols of dNTP and Taq DNA polymerase with a concentration of 5U/mu L and contain specific primer pairs of respective 4 species of target bacteria in an amount of 10 mu M each.; The detection kit and the detection method of the invention can detect the 8 species of pathogenic bacteria in the diary products with high sensitivity, is short in detection time, simple and quick in operation, and can save a large amount of labor and financial resources and meet requirements for quick detection.

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乳制品中 8 种致病菌检测试剂盒及其检测方法

[57] 摘要

用于食品微生物污染监控及检测的乳制品中 8 种致病菌检测试剂盒及其检测方法。所述的试剂盒包括检测溶液 A 和检测溶液 B；检测溶液 A 和 B 中均含 10mM Tris·Cl、50mM KCl、25mM $MgCl_2$、dNTP 各 2.5mM 及 Taq DNA 聚合酶 5U/μL，还分别各自含 4 种目标菌的特异性引物对各 10μM。使用本发明的检测试剂盒及检测方法，可以高灵敏度地检测乳制品中 8 种致病菌，并且检测耗时短，操作简捷，可以节省大量的劳力和财力，适合快速检测的要求。

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1、乳制品中 8 种致病菌检测试剂盒，其特征在于包括检测溶液 A 和检测溶液 B；

其中，检测溶液 A 中含 10 mM Tris·Cl、50 mM KCl、25mM $MgCl_2$、dNTP 各 2.5 mM、Taq DNA 聚合酶 5U/μL 及阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌的引物对各 10 μM；

检测溶液 B 中含 10 mM Tris·Cl、50 mM KCl、25mM $MgCl_2$、dNTP 各 2.5 mM、Taq DNA 聚合酶 5U/μL 及致病性嗜水气单胞菌、奇异变形杆菌、肺炎克雷伯氏菌和气味沙雷氏菌的引物对各 10 μM；

其中，所述 8 种致病菌的特异性引物序列如下：

菌株	上、下游引物序列（5′-3′）
阪崎肠杆菌	GGGTTGTCTGCGAAAGCGAA GTCTTCGTGCTGCGAGTTTG
粘质沙雷氏菌	GGAAGCGACTGCATTATTACCAT TGGTGGTGGCGATCACTT
绿脓杆菌（ETA 基因）	GACAACGCCCTCAGCATCACCAGC CGCTGGCCCATTCGCTCCAGCGCT
普通变形杆菌	GATGGCAAGTACAAGTAAG GACGCTGAGATTGACCTA
奇异变形杆菌	CAACGTGAGATTAGTGGTGA CTGCTTTAAGTTCACAAATTAAGTG
肺炎克雷伯氏菌	TGGCCCGCGCCCAGGGTTCGAAA GATGTCGTCATCGTTGATGCCGAG
气味沙雷氏菌	TCGAGCGGTAGCACAGGA CAACGTATTAAGCTATTG
致病性嗜水气单胞菌（aerA 基因）	CCAAGGGGTCTGTGGCGACA TTTCACCGGTAACAGGATTG

2、乳制品中 8 种致病菌的检测方法，其特征在于使用权利要求 1 所述的试剂盒，包括如下步骤：

① 取 1ul 待测样品 DNA 溶液，加入 10ul 检测溶液 A 和 14ul 灭菌超纯水，

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总体积 25ul；另取 1ul 待测样品 DNA 溶液，加入 10ul 检测溶液 B 和 14ul 灭菌超纯水，总体积 25ul；两个反应体系分别在 5000 r/min 离心 10s，然后按下列参数分别进行 PCR 扩增：

预变性：94℃，3 min；

进入循环：94℃变性 60s，56℃退火 60s，72℃延伸 60s，35 次循环；

终止延伸：72℃，7 min；

② 将 PCR 扩增产物进行 DHPLC 分析：

色谱柱：PS-DVB & C18 DNASep 色谱柱，4.6 mm×50 mm，粒度 3 μm；

柱温：50℃；

流动相（体积比）：0.0 min，51.0% 缓冲溶液 A，49.0% 缓冲溶液 B；

0.5 min，45.7% 缓冲溶液 A，54.3% 缓冲溶液 B；

2.7 min，41.3% 缓冲溶液 A，58.7% 缓冲溶液 B；

4.9 min，38.7% 缓冲溶液 A，61.3% 缓冲溶液 B；

7.1 min，37.1% 缓冲溶液 A，62.9% 缓冲溶液 B；

9.3 min，35.9% 缓冲溶液 A，64.1% 缓冲溶液 B；

其中，缓冲溶液 A 是浓度 0.1mM 的 TEAA 水溶液；缓冲溶液 B 是浓度为 0.1M TEAA 水溶液与乙腈按体积比 3:1 的混合溶液；

流速：0.9 mL/min；

检测器：荧光检测器，光源：150W Xenon 灯；激发谱带宽：15 nm；发射谱带宽：15.3 nm；检测灵敏度：在波长 350 nm 积分 2 s；

上样量：PCR 产物 5μL。

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乳制品中8种致病菌检测试剂盒及其检测方法

技术领域

本发明涉及乳制品中 8 种致病菌检测试剂盒及其检测方法，尤其涉及利用 mPCR-DHPLC 技术进行检测的检测方法及所使用的试剂。。

背景技术

2004 年，安徽阜阳劣质奶粉哺喂的“大头娃娃”事件曝光后，奶粉安全已成为全社会的焦点，2008 年因婴幼儿配方奶粉中三聚氰胺问题而引发的婴幼儿疾病的事件，更使全世界的目光都聚焦在奶粉等的食用安全方面。针对国内外不同品牌奶粉和进口原料奶粉中对婴幼儿有害病原菌的污染情况进行调研，发现奶粉中污染坂崎肠杆菌、肺炎克雷伯氏菌(*Klebsiella Pnaumoniae*)、变形杆菌(*Proteuse*)、金黄色葡萄球菌、大肠杆菌、绿脓杆菌(*Pseudomonas aeruginosa*)、溶血性链球菌(*Streptococcus*)、粘质沙雷氏菌(*serratia macescens*)、气味沙雷氏菌(*serratia odorifera*)等的污染几率较高。奶粉是母乳的替代品，婴幼儿的主要食物来源，奶粉安全意义重大。

阪崎肠杆菌(*Enterobacter sakazakii*)是人和动物肠道内寄生的一种革兰阴性无芽孢杆菌，属肠杆菌科的一种。该菌在一定条件下可引起人和动物致病，因此被称为条件致病菌。1961 年，英国首次报道 2 例由阪崎肠杆菌引起的脑膜炎病例，以后相继在美国、希腊、荷兰、冰岛、加拿大、比利时等国家报道了新生儿阪崎肠杆菌感染事件。具不完全资料报道，阪崎肠杆菌能引起严重的新生儿脑膜炎、小肠结肠炎和菌血症，并且可能引起神经系统后遗症和死亡，死亡率高达 50%以上。2001 年 4 月美国田纳西州发生阪崎肠杆菌感染事件后，医务人员与疾病控制中心协同对感染源进行了调查，结果从 10 例婴儿体内分离到的阪崎肠杆菌，与从婴儿所食用的已开罐和未开罐的婴儿配方奶粉中分离到的指纹图谱一致。由此导致国际间第一次因阪崎肠杆菌污染而致商业婴儿配方粉的广泛召回。目前，虽然还不能确定阪崎肠杆菌的宿主和转播途径，但多起新生儿阪崎肠杆菌感染事件基本证实了婴儿配方奶粉是主要感染源。

变形杆菌属有 4 个种：普通变形杆菌、奇异变形杆菌、产粘变形杆菌和潘

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氏变形杆菌，其中以普通变形杆菌和奇异变形杆菌与临床关系较密切，特别是奇异变形杆菌可引起败血症，病死率较高。近年来，有关变形杆菌引起食物中毒的事件有所增加。

绿脓杆菌又称铜绿假单胞菌，属于假单胞菌属，在自然界中分布广泛，多见于水、空气、土壤中和物体表面上，也可见于正常人体表及人和动物的肠道中，人、畜肠道是绿脓杆菌的繁殖场所，为环境的主要污染源之一，也是一种重要的条件致病菌，常引起人皮肤化脓感染，特别是烧伤、烫伤、眼部疾病患者被感染后，常使病情恶化，并可引起败血症。2004 年也见从卫生巾中分离出绿脓杆菌的报道。国家质量监督检验检疫总局、卫生部等部门有明确规定，在食品、化妆品、外用药品以及絮用纤维制品、妇女卫生用品等物品中不得检出绿脓杆菌。传统的检测方法主要依靠绿脓杆菌的生物学特征，通过分离培养结合生化鉴定等方法进行检测，不仅操作繁琐、耗时长，且容易延误诊断。

目前国内外对微生物的检测方法还多停留在检测效率低、目标单一的水平上，还主要依靠传统的增菌、分离、生化鉴定方法，对于产芽孢菌的检测则更为落后。一般说来鉴定一种细菌需要 7～10 天，一些生化特性复杂的细菌（如致病性嗜水气单胞菌）最长的检测周期可达 20 天之久，这就影响了检测鉴定的周期。该现状显然不能满足目前食品安全迅速发展的需求。即便现在已经发展起来了 PCR 和实时 PCR 技术，也仅可达到对已知的常规微生物进行检测。而对于混合微生物样本、不常见的、苛刻的、发生变异的细菌则缺乏稳定而可靠的鉴定手段。致病性嗜水气单胞菌等对培养条件要求较高，生长缓慢，检测和鉴定周期长，而且传统培养生化检测方法检出率不高。因此，建立针对乳制品中多种微生物的高效、灵敏、便捷的检测方法很有必要。

发明内容

本发明的目的在于提供用于乳制品中 8 种致病菌的检测试剂盒及其 mPCR-DHPLC 检测方法，这几种乳制品中的常见致病菌分别是阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌、普通变形杆菌、奇异变形杆菌、肺炎克雷伯氏菌、气味沙雷氏菌、致病性嗜水气单胞菌。

首先，本发明所述的乳制品中 8 种致病菌检测试剂盒，该试剂盒包括检测溶液 A 和检测溶液 B；

其中，检测溶液 A 中含 10 mM Tris·Cl、50 mM KCl、25mM $MgCl_2$、dNTP

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混合物（包括 dATP、dGTP、dCTP 和 dTTP 各 2.5 mM）、Taq DNA 聚合酶 5U/μL 及阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌的引物对各 10 μM；

检测溶液 B 中含 10 mM Tris·Cl、50 mM KCl、25mM $MgCl_2$、dNTP 混合物（包括 dATP、dGTP、dCTP 和 dTTP 各 2.5 mM）、Taq DNA 聚合酶 5U/μL 及致病性嗜水气单胞菌、奇异变形杆菌、肺炎克雷伯氏菌和气味沙雷氏菌的引物对各 10 μM；

其中，所述 8 种致病菌的特异性引物序列如下：

菌株	上、下游引物序列（5′-3′）	扩增片段
阪崎肠杆菌	GGGTTGTCTGCGAAAGCGAA GTCTTCGTGCTGCGAGTTTG	228bp
粘质沙雷氏菌	GGAAGCGACTGCATTATTACCAT TGGTGGTGGCGATCACTT	332bp
绿脓杆菌（ETA 基因）	GACAACGCCCTCAGCATCACCAGC CGCTGGCCCATTCGCTCCAGCGCT	396 bp
普通变形杆菌	GATGGCAAGTACAAGTAAG GACGCTGAGATTGACCTA	413 bp
奇异变形杆菌	CAACGTGAGATTAGTGGTGA CTGCTTTAAGTTCACAAATTAAGTG	241 bp
肺炎克雷伯氏菌	TGGCCCGCGCCCAGGGTTCGAAA GATGTCGTCATCGTTGATGCCGAG	368bp
气味沙雷氏菌	TCGAGCGGTAGCACAGGA CAACGTATTAAGCTATTG	412bp
致病性嗜水气单胞菌（aerA 基因）	CCAAGGGGTCTGTGGCGACA TTTCACCGGTAACAGGATTG	209 bp

本发明还提供了利用上述试剂盒检测乳制品中 8 种致病菌的检测方法，包括如下步骤：

① 取 1ul 待测样品 DNA 溶液，加入 10ul 检测溶液 A 和 14ul 灭菌超纯水，总体积 25ul；另取 1ul 待测样品 DNA 溶液，加入 10ul 检测溶液 B 和 14ul 灭菌超纯水，总体积 25ul；两个反应体系分别在 5000 r/min 离心 10s，然后按下列参数分别进行 PCR 扩增：

预变性：94℃，3 min；

进入循环：94℃变性 60s，56℃退火 60s，72℃延伸 60s，35 次循环；

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终止延伸：72℃，7 min；

② 将 PCR 扩增产物进行 DHPLC 分析：

色谱柱：PS-DVB & C18 DNASep 色谱柱，4.6 mm×50 mm，粒度 3 μm；

柱温：50℃；

流动相（体积比）：0.0 min，51.0% 缓冲溶液 A，49.0% 缓冲溶液 B；

0.5 min，45.7% 缓冲溶液 A，54.3% 缓冲溶液 B；

2.7 min，41.3% 缓冲溶液 A，58.7% 缓冲溶液 B；

4.9 min，38.7% 缓冲溶液 A，61.3% 缓冲溶液 B；

7.1 min，37.1% 缓冲溶液 A，62.9% 缓冲溶液 B；

9.3 min，35.9% 缓冲溶液 A，64.1% 缓冲溶液 B；

其中，缓冲溶液 A 是浓度 0.1mM 的 TEAA 水溶液；缓冲溶液 B 是浓度为 0.1M TEAA 水溶液与乙腈按体积比 3:1 的混合溶液；

流速：0.9 mL/min；

检测器：荧光检测器，光源：150W Xenon 灯；激发谱带宽：15 nm；发射谱带宽：15.3 nm；检测灵敏度：在波长 350 nm 积分 2 s；

上样量：PCR 产物 5μL。

检测结束后，根据 DHPLC 检测图谱中特征色谱峰的峰形、保留时间以及与阳性对照谱图的对照，来确定样品的染菌情况。

本发明采用 mPCR-DHPLC 技术，针对乳制品中 8 种致病菌建立灵敏便捷的检测方法，并建立应用于该方法的快速检测试剂盒。使用本发明的检测试剂盒及检测方法，可以高灵敏度地检测乳制品中 8 种致病菌，并且检测耗时短，操作简捷，可以节省大量的劳力和财力，适合快速检测的要求。

附图说明

本发明附图 7 幅：

图 1 是阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌单一菌 PCR-电泳检测结果；

图 2 是阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌四重 PCR-电泳检测电泳图；

图 3 是阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌的四重 PCR-DHPLC 检测谱图；

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图 4 是奇异变形杆菌、肺炎克雷伯氏菌、气味沙雷氏菌和致病性嗜水气单胞菌单一菌 PCR-电泳检测结果；

图 5 是奇异变形杆菌、肺炎克雷伯氏菌、气味沙雷氏菌和致病性嗜水气单胞菌四重 PCR-电泳检测电泳图；

图 6 是奇异变形杆菌、肺炎克雷伯氏菌、气味沙雷氏菌和致病性嗜水气单胞菌四重 PCR-DHPLC 检测谱图；

图 7 是阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌 mPCR-DHPLC 灵敏度试验结果；

图 8 是奇异变形杆菌、肺炎克雷伯氏菌、气味沙雷氏菌和致病性嗜水气单胞菌 mPCR-DHPLC 灵敏度试验结果；

上述附图中，横坐标均为保留时间（单位：分钟 min），纵坐标表示吸收峰信号强度（单位：毫伏 mV）。

具体实施方式

下面为本发明的具体实施例，其对本方法的建立及其应用作进一步的说明，但并不以任何形式限制本发明的内容。

本部分试验所用的营养肉汤培养液，普通琼脂培养平板，以及所使用的各菌株的增菌、分离和生化鉴定培养基等均按照相关的国家标准（GB）、检验检疫行业标准（SN）或国际权威标准方法中的配方，购于美国 BD 公司。

细菌基因组 DNA 提取试剂（TakaRa MiniBEST Bacterial Genomic DNA Extraction kit）；Taq DNA 聚合酶，10×PCR buffer，dNTPs 等试剂购自宝生物工程（大连）有限公司；三乙胺乙酰盐（TEAA，色谱纯）购自 Transgenomic 公司；乙腈（色谱纯）购自 Fisher 公司。

本部分试验所用的主要仪器和设备包括基因扩增仪（美国 ABI 公司）和变性高效液相色谱（简称 DHPLC，美国 Transgenomic 公司）。

本研究所用标准菌株均购自美国典型菌种保藏中心（ATCC）和中国医学微生物菌种保藏管理中心（CMCC）。

实施例 1、乳制品中 8 种致病菌检测试剂盒及其检测方法的建立

（1）引物的设计、合成与试剂盒的组装：

菌株	上、下游引物序列（5′-3′）	扩增片段

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阪崎肠杆菌	GGGTTGTCTGCGAAAGCGAA GTCTTCGTGCTGCGAGTTTG	228bp
粘质沙雷氏菌	GGAAGCGACTGCATTATTACCAT TGGTGGTGGCGATCACTT	332bp
绿脓杆菌（ETA 基因）	GACAACGCCCTCAGCATCACCAGC CGCTGGCCCATTCGCTCCAGCGCT	396 bp
普通变形杆菌	GATGGCAAGTACAAGTAAG GACGCTGAGATTGACCTA	413 bp
奇异变形杆菌	CAACGTGAGATTAGTGGTGA CTGCTTTAAGTTCACAAATTAAGTG	241 bp
肺炎克雷伯氏菌	TGGCCCGCGCCCAGGGTTCGAAA GATGTCGTCATCGTTGATGCCGAG	368bp
气味沙雷氏菌	TCGAGCGGTAGCACAGGA CAACGTATTAAGCTATTG	412bp
致病性嗜水气单胞菌（aerA 基因）	CCAAGGGGTCTGTGGCGACA TTTCACCGGTAACAGGATTG	209 bp

在此基础上，设计用于乳制品中 8 种致病菌检测的试剂盒，该试剂盒包括两组检测溶剂：检测溶液 A 和检测溶液 B；

其中，检测溶液 A 中含 10 mM Tris·Cl、50 mM KCl、25mM $MgCl_2$、dNTP 混合物（包括 dATP、dGTP、dCTP 和 dTTP 各 2.5 mM）、Taq DNA 聚合酶 5U/μL 及阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌的引物对各 10 μM；

检测溶液 B 中含 10 mM Tris·Cl、50 mM KCl、25mM $MgCl_2$、dNTP 混合物（包括 dATP、dGTP、dCTP 和 dTTP 各 2.5 mM）、Taq DNA 聚合酶 5U/μL 及致病性嗜水气单胞菌、奇异变形杆菌、肺炎克雷伯氏菌和气味沙雷氏菌的引物对各 10 μM。

（2）mPCR-DHPLC 检测方法

本检测方法使用本实施例建立的检测试剂盒，包括如下检测步骤：

① 待检样品的制备：

a. 奶制品样品：无菌方法称取 100 克样品，加到已预热的（44℃）装有 900mL 改良月桂基硫酸盐胰蛋白胨肉汤（mLST）的 2L 三角瓶中，振摇使样品充分混匀后，44℃恒温培养 22-24h。在液面 1cm 以下取 mLST 增菌液 0.2mL 加到装有 5mL 心脑浸液肉汤（BHI）的小试管中，混匀，36℃恒温水浴 4h，水面要高于

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试管中培养基高度。取 BHI 增菌液 1.5mL，使用细菌基因组提取试剂盒提取其基因组 DNA，制取 PCR 模板。标记，直接作为 PCR 模板或-20℃保存。

b. 标准菌株：挑取单个菌落于营养肉汤中培养 18～24h，使用细菌基因组提取试剂盒提取其基因组 DNA，制取 PCR 模板。标记，直接作为 PCR 模板或-20℃保存。；

② PCR 扩增：

取 1ul 待测样品 DNA 溶液，加入 10ul 检测溶液 A 和 14ul 灭菌超纯水，总体积 25ul；另取 1ul 待测样品 DNA 溶液，加入 10ul 检测溶液 B 和 14ul 灭菌超纯水，总体积 25ul；两个反应体系分别在 5000 r/min 离心 10s，然后按下列参数分别进行 PCR 扩增：

预变性：94℃，3 min；

进入循环：94℃变性 60s，56℃退火 60s，72℃延伸 60s，35 次循环；

终止延伸：72℃，7 min；

③ DHPLC 分析：

色谱柱：PS-DVB & C18 DNASep 色谱柱，4.6 mm×50 mm，粒度 3 μm；

柱温：50℃；

流动相（体积比）：0.0 min，51.0% 缓冲溶液 A，49.0% 缓冲溶液 B；
0.5 min，45.7% 缓冲溶液 A，54.3% 缓冲溶液 B；
2.7 min，41.3% 缓冲溶液 A，58.7% 缓冲溶液 B；
4.9 min，38.7% 缓冲溶液 A，61.3% 缓冲溶液 B；
7.1 min，37.1% 缓冲溶液 A，62.9% 缓冲溶液 B；
9.3 min，35.9% 缓冲溶液 A，64.1% 缓冲溶液 B；

其中，缓冲溶液 A 是浓度 0.1mM 的 TEAA 水溶液；缓冲溶液 B 是浓度为 0.1M TEAA 水溶液与乙腈按体积比 3:1 的混合溶液；

流速：0.9 mL/min；

检测器：荧光检测器，光源：150W Xenon 灯；激发谱带宽：15 nm；发射谱带宽：15.3 nm；检测灵敏度：在波长 350 nm 积分 2 s；

上样量：PCR 产物 5μL。

实施例 2、与常规 PCR-电泳检测方法的比较

实施例 1 所建立的检测试剂盒及检测方法涉及两组 PCR 体系，第一组复合

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PCR 体系一次检测阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌；第二组复合 PCR 体系一次检测致病性嗜水气单胞菌、奇异变形杆菌、肺炎克雷伯氏菌和气味沙雷氏菌。

按照实施例 1 所建立的方法提取标准菌株的 DNA 作为模板，按照实施例 1 所建立的方法的 PCR 条件进行扩增：第一组阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌四种菌的预期扩增片段分别为：228bp、332bp、396 bp、413 bp；第二组致病性嗜水气单胞菌、奇异变形杆菌、肺炎克雷伯氏菌和气味沙雷氏菌四种菌的预期扩增片段分别为：209bp、241bp、368bp、412bp；对扩增后的扩增产物分别采用电泳及实施例 1 所建立的 DHPLC 检测条件检测。

第一组阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌各自单一 PCR 扩增的电泳检测结果见附图 1 所示；四重 PCR 扩增的电泳结果见附图 2 所示；四重 PCR 扩增的 DHPLC 检测结果见附图 3 所示；第二组致病性嗜水气单胞菌、奇异变形杆菌、肺炎克雷伯氏菌和气味沙雷氏菌各自单一 PCR 扩增的电泳检测结果见附图 4 所示；四重 PCR 扩增的电泳结果见附图 5 所示；四重 PCR 扩增的 DHPLC 检测结果见附图 6 所示。

由图 1～3 可见，当第一组四种细菌作单一 PCR 扩增和电泳检测时，均能明显地检测出各自单一的条带；而当该四种细菌作复合 PCR 扩增和电泳检测时，396 bp、413 bp 的扩增条带却无法分开；但 DHPLC 检测中它们能清晰地分离并被准确检测；由图 4～6 可见，当第二组四种细菌作单一 PCR 扩增和电泳检测时，均能明显地检测出各自单一的条带；而当该四种细菌作复合 PCR 扩增和电泳检测时，209 bp 和 241 bp 的扩增条带很难分开，368bp、412bp 的扩增条带很难分开，但 DHPLC 检测中它们能清晰地分离并被准确检测。由此可见，本发明实施例 1 所建立的 mPCR-DHPLC 有更好的检测效果。

实施例 3、PCR-DHPLC 检测方法特异性试验

取表 1 中所列试验菌种，经培养后分别提取基因组 DNA，建立模板库。然后以这些基因组 DNA 为模板，采用实施例 1 所建立的方法进行 PCR-DHPLC 分析检测。

表 1

菌株名称	拉丁名

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普通变形杆菌	*Proteus vulgaris*
奇异变形杆菌	*Proteus mirabilis*
致病性嗜水气单胞菌	*Aeromonas hydrophila*
非致病性嗜水气单胞菌	*Aeromonas hydrophila*
类志贺邻单胞菌	*Plesiomonas shigelloides*
副溶血性弧菌	*Vibrio parahaemolyticus*
副溶血性弧菌	*Vibrio parahaemolyticus*
副溶血性弧菌	*Vibrio parahaemolyticus*
溶藻性弧菌	*Vibrio alginolyticus*
创伤弧菌	*Vibrio vulnificus*
拟态弧菌	*Vibrio mimicus*
拟态弧菌	*Vibrio mimicus*
梅氏弧菌	*Vibrio metschnikouii*
河流弧菌	*Vibrio fluvialis*
单核细胞增生李斯特氏菌	*Listeria monocytogenes*
单核细胞增生李斯特氏菌	*Listeria monocytogenes*
单核细胞增生李斯特氏菌	*Listeria monocytogenes*
绵羊李斯特氏菌	*Listeria ivanovii*
英诺克李斯特氏菌	*Listeria innocua*
威尔斯李斯特氏菌	*Listeria welshimeri*
西尔李斯特氏菌	*Listeria seeligeri*
格氏李斯特氏菌	*Listeria grayi*
脱氮李斯特氏菌	*listeria denitrificans*
沙门氏菌	*Salmonella*
鼠伤寒沙门氏菌	*Salmonella. typhimurium*
甲型副伤寒沙门氏菌	*Salmonella. paratyphi A*
乙型副伤寒沙门氏菌	*Salmonella. paratyphi B*
丙型副伤寒沙门氏菌	*Salmonella. paratyphi C*
猪霍乱沙门氏菌	*Salmonella enterica subsp.*
猪霍乱沙门氏菌	*Salmonella enterica subsp.*
猪霍乱沙门氏菌	*Salmonella. cholerae*
肠炎沙门氏菌	*Salmonella. enteritidis*
亚利桑那沙门氏菌	*Salmonella enterica subsp. arizonae*
阿巴特图巴沙门氏菌	*Salmonella enterica subsp. enterica serovar Abaetetuba*
汤卜逊沙门氏菌	*Salmonella.thompson*
圣保罗沙门氏菌	*Salmonella.taint-paul*

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海德堡沙门氏菌	*Salmonella.heidelberg*
都柏林沙门氏菌	*Salmonella dublin*
绿脓杆菌（铜绿假单胞菌）	*Pseudomonas aeruginosa*
绿脓杆菌（铜绿假单胞菌）	*Pseudomonas aeruginosa*
荧光假单胞菌	*Pseudomonas fluorescens*
恶臭假单胞菌	*Pseudomonas putida*
产碱假单胞菌	*Pseudomonas alcaligenes*
类产碱假单胞菌	*Pseudomonas pseodoalcaligenes*
洋葱假单胞菌	*Pseudomonas cepacia*
绿针假单胞菌	*Pseudomonas choloraphis*
稻草假单胞菌	*Pseudomonas straminea*
皮氏假单胞菌	*Pseudomonas pickettii*
门多萨假单胞菌	*Pseudomonas mendocina*
施氏假单胞菌	*Pseudomonas stutzeri*
阪崎肠杆菌	*Enterobacter.sakazakii*
大肠埃希氏菌	*Escherichia coli*
大肠埃希氏菌	*Escherichia coli*
产肠毒素大肠埃希氏菌	*Enterotoxigenic E. coli*
肠致病性大肠埃希氏菌	*Enteropathogenic E.coli*
肠侵袭性大肠埃希氏菌	*Enteroinvasive E.coli*
摩根氏摩根氏菌	*Morganella morganii*
产酸克雷伯氏菌	*Klebsiella oxytoca*
肺炎克雷伯氏菌	*Klebsiella peneumoniae*
弗劳地柠檬酸杆菌	*Citrobacter freundii*
弗地劳枸橼酸杆菌	*Citrobacter freundii*
弗劳地柠檬酸杆菌	*Citrobacter freundii*
阴沟肠杆菌	*Enterobacter cloacae*
阪崎肠杆菌	*Enterobacter.sakazakii*
产气肠杆菌	*Enterobacter aerogenes*
福氏志贺氏菌	*Shigella flexneri*
金黄色葡萄球菌	*Staphylococcus aureus*
金黄色葡萄球菌	*Staphylococcus aureus subsp. aureus*
小肠结肠炎耶尔森氏菌	*Yersinia enterocolitica*
溶血性链球菌	*Streptococcus hemolyticus*

特异性试验结果表明，采用实施例 1 所建立的检测试剂盒及检测方法，两

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次检测即可特异地检测 8 种目标致病菌；采用检测试剂 A 进行的检测，只有阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌检测出特异样品峰；采用检测试剂 B 进行的检测，只有致病性嗜水气单胞菌、奇异变形杆菌、肺炎克雷伯氏菌和气味沙雷氏菌检测出特异样品峰，其它对照细菌的检测结果都为阴性，没有假阳性和假阴性结果，具有很高的特异性。

实施例 4、PCR-DHPLC 模拟污染样品灵敏度试验

以奶粉为例，制备乳制品中微生物模拟污染样品，用比浊法定量所模拟污染的菌液浓度，单位为 CFU/mL。将致病菌的模拟污染肉汤进行系列稀释得到 10^{-3}、10^{-4}、10^{-5}、10^{-6}、10^{-7}、10^{-8}、10^{-9}、10^{-10} 的菌液，然后采用实施例 1 所建立的试剂盒及方法提取 DNA，各取 2 μL 做为模板，同样按照实施例 1 的方法进行 mPCR-DHPLC 检测，将检测结果与稀释梯度结果比较，确定检测灵敏度。检测结果如附图 7（第一组）和附图 8（第二组）所示。

表 1 和表 2 为致病菌在不同稀释度下的细菌数量。

表 1 第一组模拟污染液梯度稀释细菌数量表

序号	
L01	阪崎肠杆菌 29000\粘质沙雷氏菌 51000\绿脓杆菌 35000\普通变形杆菌 17000
L02	阪崎肠杆菌 2900\粘质沙雷氏菌 5100\绿脓杆菌 3500\普通变形杆菌 1700
L03	阪崎肠杆菌 290\粘质沙雷氏菌 510\绿脓杆菌 350\普通变形杆菌 170
L04	阪崎肠杆菌 29\粘质沙雷氏菌 51\绿脓杆菌 35\普通变形杆菌 17

表 2 第二组模拟污染液梯度稀释细菌数量表

序号	
L05	奇异变形杆菌 16000\肺炎克雷伯氏菌 40000\ 气味沙雷氏菌 29000\致病性嗜水气单胞菌 120000
L06	奇异变形杆菌 1600\肺炎克雷伯氏菌 4000\ 气味沙雷氏菌 2900\致病性嗜水气单胞菌 12000
L07	奇异变形杆菌 160\肺炎克雷伯氏菌 400\ 气味沙雷氏菌 290\致病性嗜水气单胞菌 1200
L08	奇异变形杆菌 16\肺炎克雷伯氏菌 40\ 气味沙雷氏菌 29\致病性嗜水气单胞菌 120

如附图 7 所示，第一组可以检测到表 2 中 L03 梯度的阪崎肠杆菌 290 CFU/mL，粘质沙雷氏菌 510 CFU/mL，绿脓杆菌 350 CFU/mL，普通变形杆菌 170 CFU/mL，即可以检测到 10^2 的数量级的菌浓度；而 PCR-凝胶电泳却只能检

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测到L02梯度（10^3的数量级）的菌浓度。

如附图 8 所示，第二组可以检测到表 2 中 L07 梯度的奇异变形杆菌 160 CFU/mL，肺炎克雷伯氏菌 400 CFU/mL，气味沙雷氏菌 290 CFU/mL，致病性嗜水气单胞菌 1200 CFU/mL，即可以检测到10^2的数量级的菌浓度；而 PCR-凝胶电泳却只能检测到L06梯度（10^3的数量级）的菌浓度。

结果表明，本研究所建立的乳粉中致病菌 PCR-DHPLC 高通量检测方法的灵敏度非常高，可以达到10^2 CFU/mL 的数量级。

实施例 5、应用于实际样品的检测试验

自 2007 年 12 月至 2008 年 6 月的 7 个月期间，采用实施例 1 建立的多重 PCR-DHPLC 方法快速筛选，同时采用检验检疫行业标准（SN/T 1632.1-3-2005、SN/T 0751-1999、SN/T 0738-1997）的培养鉴定方法进行验证比较，同时检测实际样品，共检测 2675 份乳制品样品。结果表明，用多重 PCR-DHPLC 方法筛选检出的 21 份阳性样本，其中检出普通变形杆菌 13 份，奇异变形杆菌 3 份，4 份样品检出阪崎肠杆菌，1 份样品检出粘质沙雷氏菌，采用经典的生化方法进行验证，均证实多重 PCR-DHPLC 方法检测准确率 100%。

本实施例中，采用检验检疫行业标准 SN/T 1632.1-3-2005、SN/T 0751-1999、SN/T 0738-1997）的方法，对 8 种菌均进行检测，平均总耗时（包括样品制备）576 h，检测耗时（不包括样品制备）575 h；使用 mPCR-DHPLC 方法，检测每个样品平均总耗时（包括样品制备）29 h，检测耗时（不包括样品制备）0.5 h。

实施例 6、应用于实际阳性样品的检测试验

对既往检测工作中，已经由行标检测确认为阳性的样品应用实施例 1 的 PCR-DHPLC 方法进行检测。

（1）在普通变形杆菌和奇异变形杆菌阳性样品检测中的应用

① 乳制品中普通变形杆菌和奇异变形杆菌检测结果

留存样品中，有 3 份样品已证实同时检出普通变形杆菌和奇异变形杆菌。用实施例 1 建立的方法检测这 3 份样品，经多重 PCR-DHPLC 检出 3 份样品普通变形杆菌和奇异变形杆菌阳性。

本试验采用普通变形杆菌和奇异变形杆菌阳性标准菌株、大肠杆菌阴性对照菌株（ATCC 17803）及水空白对照进行检测的结果表明，阳性对照检测出

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DHPLC 的阳性吸收峰，且出峰时间和峰型重现性较好，阴性及水对照结果未见 DHPLC 吸收峰。多重 PCR-DHPLC 同时检测 3 株乳制品中的普通变形杆菌和奇异变形杆菌，均具有很好的阳性吸收峰重现性。

② 乳制品中普通变形杆菌和奇异变形杆菌分离株常规鉴定结果

5 株乳制品中的普通变形杆菌和奇异变形杆菌，常规分离培养鉴定结果与 PCR-DHPLC 检测结果比较，符合率为 100 %。

结果表明：本实施例 1 建立的多重 PCR-DHPLC 检测乳制品致病菌的方法，与经典的培养生化鉴定方法具有很好的符合性和结果重现性。

（2）在普通变形杆菌和阪崎肠杆菌阳性样品检测中的应用

留存样品中，其中有 2 份样品已证实同时检出普通变形杆菌和阪崎肠杆菌。用实施例 1 建立的方法检测这 2 份样品，经多重 PCR-DHPLC 检出 2 份样品普通变形杆菌和阪崎肠杆菌阳性。取从样品中分离出的所留存的普通变形杆菌和阪崎肠杆菌分离株，提取 DNA 进行 PCR-DHPLC 的验证试验，普通变形杆菌和阪崎肠杆菌均经 DHPLC 检测到了扩增吸收峰，且出峰时间和峰型重现性较好，以大肠杆菌为阴性对照的检测结果则为阴性。

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SEQUENCE LISTING

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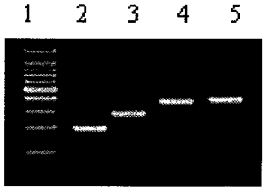


图 1



图 2

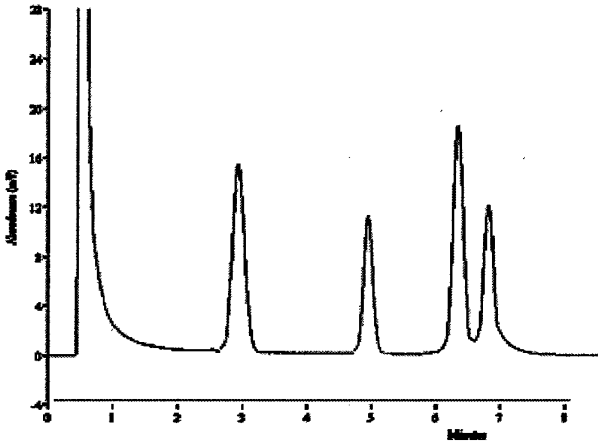


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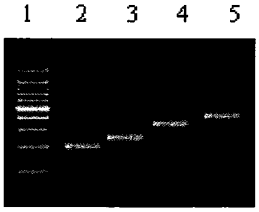


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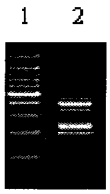


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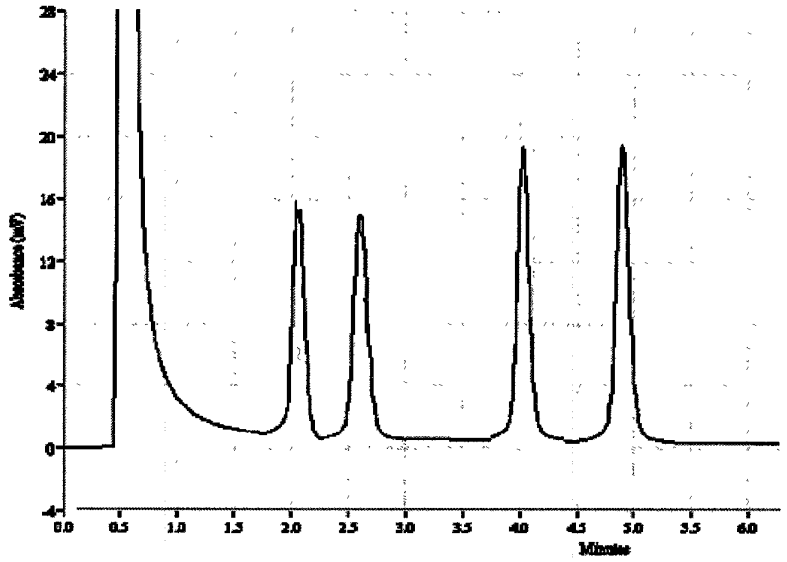


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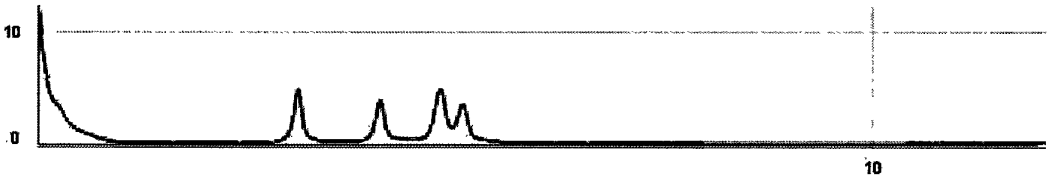


图 7

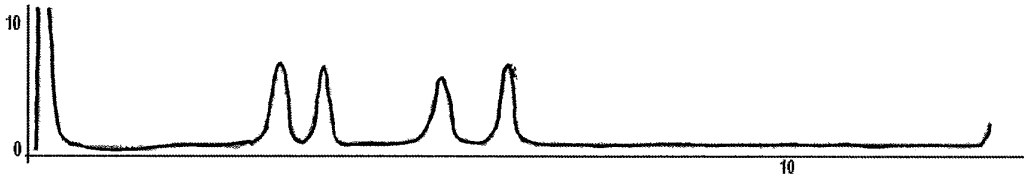


图 8

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(54) **HOMOGENEOUS MULTIPLEX SCREENING ASSAYS AND KITS**

HOMOGENE MULTIPLEX-SCREENING-TESTS UND KITS

DOSAGES BIOLOGIQUES MULTIPLEXES HOMOGENES ET KITS ASSOCIES

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WO-A-01/71044
WO-A-03/100101
WO-A1-03/020967
WO-A2-02/100157
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US-B1- 6 738 502

- **LEE L.G. ET AL.: 'Seven color homogeneous detection of six PCR products' BIOTECHNIQUES vol. 27, no. 2, 1999, pages 342 - 349, XP000851393**
- **HAN M. ET AL.: 'Quantum dot tagged microbeads for multiplexed optical coding of biomolecules' NATURE BIOTECHNOLOGY vol. 19, 2001, pages 631 - 635, XP001069134**
- **RIED T. ET AL.: 'Simultaneous visualization of seven different DNA probes by in situ hybridization using combinatorial fluorescence and digital imaging microscopy' PNAS vol. 89, 1992, pages 1388 - 1392, XP002073155**
- **TYAGI S. ET AL: 'Wavelength-shifting molecular beacons' NATURE BIOTECHNOLOGY vol. 18, 2000, pages 1191 - 1196, XP002215066**

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EP 1 629 108 B1

Description

TECHNICAL FIELD

[0001] 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

[0002] 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 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.

[0003] 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 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.

[0004] WO-A-03/020967 disclosed a method for determining at least three analytes, wherein one probe has a first label, a second probe has a second label, and a third probe has two sub-populations: one having the first label, the other having the second label. The analytes are detected according to the signal intensities determined for the first and second labels. The system may be extended to more analytes as exemplified by a six analyte system using three labels.

[0005] 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

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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.

[0006] An aspect of this invention is highly multiplexed homogeneous assays for screening samples for the presence of at least six target nucleic acid sequences, utilizing conventional fluorescence detection equipment and techniques, and fluorescently labeled hybridization probes.

[0007] Another aspect of this invention is kits for carrying out particular screening assays according to this invention.

SUMMARY

[0008] 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.

[0009] 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.

[0010] 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.

[0011] 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.

[0012] 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.

[0013] 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 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

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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.

[0014] 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.

[0015] 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 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 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.

[0016] 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.

[0017] 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

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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.

[0018] 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, 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.

[0019] 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.

[0020] 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 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.

[0021] 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

[0022]

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

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colors of equal fluorescence intensity, one color being shared by both probes) bind to the amplified target sequences.

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

[0023] 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 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.

[0024] Assays according to this invention utilize one probe (which may be a singlemolecule 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 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 con-

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centration, 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.

[0025] "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.

[0026] Various fluorophores can be included in panels of fluorophores. Certain fluorophores have relatively narrow emission spectra, for example, Vic, Alexa 488, 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.

[0027] 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.

[0028] 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 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.

[0029] 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 re-

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quire 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 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%.

[0030] 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.

[0031] 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.

[0032] 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.

[0033] 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.

[0034] 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. Nonetheless, when the second threshold is reached, the intensity curve of the common color will change slope to a higher value, signifying its commonality.

[0035] 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

[0036] In this example, a real-time PCR amplification assay is carried out on DNA isolated from a normally

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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 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.

[0037] 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

[0038] 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 each of the two signals unambiguously identifies each of the two bacterial species that are present in the sample.

Example 3

[0039] 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

[0040] 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 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 de-

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tect 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

[0041] 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 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

[0042] 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).

[0043] 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.

[0044] 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 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

[0045] 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

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(continued)

probe for species 9 abc

[0046] 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.

[0047] 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.

[0048] 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 scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

Claims

1. A multiplexed homogeneous *in vitro* assay to screen a sample for the presence of at least six different nucleic acid targets comprising:

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

   i) each probe in said set is labeled with a combinatorial code comprising at least two different emitter fluorophores selected from a panel of at least four spectrally differentiable fluorophores,

   ii) each target specific probe is subdivided into portions, each portion comprising a population of molecules, and wherein the number of portions is equal to the number of emitter fluorophores used to label each probe,

   iii) each portion is labeled with a different emitter fluorophore and wherein molecules of a portion are labeled with the same single emitter fluorophore,

   iv) said portions are mixed to create a single uniquely-labeled probe specific for one of said nucleic acid targets, and

   v) hybridization of each probe portion with its corresponding nucleic acid target 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 emitter 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, optionally wherein changes in emission signals are detected in real time during amplification.

3. The assay according to claim 2 wherein each probe comprises two, three or four differently labeled portions.

4. The assay according to any one of claims 1 to 3 wherein the relative amounts of the portions of each probe are adjusted relative to one another to provide balanced signal changes.

5. The assay according to any one of claims 1 to 4 wherein said set of probes additionally includes at least one probe that is ratio coded.

6. The assay according to any one of claims 1 to 5 wherein said combinatorially coded probes are quenched probes whose fluorescence is restored by hybridization to their targets, optionally 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 and preferably the fluorescence intensities of the portions of each probe are balanced relative to one another.

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7. The assay according to claim 6 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.

8. The assay according to claim 7 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, optionally wherein the relative amounts of all portions of all probes are adjusted relative to one another to provide balanced fluorescence emissions.

9. A kit of reagents for use in a multiplexed *in vitro* assay to screen a sample for the presence of at leastsix different nucleic acid targets comprising a set of at least six combinatorially coded fluorescent hybridization probes, each probe specific for a different nucleic acid target, wherein

i) each probe in said set is labeled with a combinatorial code comprising at least two different emitter fluorophores selected from a panel of at least four spectrally differentiable fluorophores,
ii) each target specific probe is subdivided into portions, each portion comprising a population of molecules, and wherein the number of portions is equal to the number of emitter fluorophores used to label each probe,
iii) each portion is labeled with a different emitter fluorophore and wherein molecules of a portion are labeled with the same single emitter fluorophore,
iv) said portions are mixed to create a single uniquely-labeled probe specific for one of said nucleic acid targets, and
v) hybridization of each probe portion with its corresponding nucleic acid target leads to a detectable fluorescent signal change indicative of its labeling.

10. The kit according to claim 9 further comprising:

(a) primers for exponential amplification of said nucleic acid targets;
(b) amplification reagents for amplifying said nucleic acid targets utilizing said primers; or
(c) at least one probe that is ratio coded.

11. The kit according to claim 9 or claim 10 wherein the combinatorially coded probes are quenched probes whose fluorescence is restored by hybridization to their targets, optionally wherein said probes are molecular beacon probes having a non-fluorescent quencher on one arm and an emitter fluorophore on the other arm.

12. The kit according to claim 11 wherein the relative amounts of the portions of each probe are adjusted relative to one another to provide balanced fluorescence emissions, optionally wherein the relative amounts of all portions of all probes are adjusted relative to one another to provide balanced fluorescence emissions.

Patentansprüche

1. Homogener Mehrfach-*in-vitro*-Assay zur Durchmusterung einer Probe auf die Anwesenheit von mindestens sechs Nukleinsäure-Targets, umfassend:

a) Inkontaktbringen der Probe mit einem Satz von mindestens sechs kombinatorisch kodierten fluoreszierenden Hybridisierungssonden, wobei jede Sonde spezifisch ist für ein anderes Nukleinsäure-Target, wobei

i) jede Sonde in dem Satz mit einem kombinatorischen Code markiert ist, der mindestens zwei verschiedene Emitterfluorophore umfasst, die ausgewählt sind aus einem Panel von mindestens vier spektral unterschiedlichen Fluorophoren,
ii) jede targetspezifische Sonde in Abschnitte unterteilt ist, wobei jeder Abschnitt eine Population von Molekülen umfasst, und wobei die Anzahl der Abschnitte gleich der Anzahl von Emitterfluorophoren ist, die verwendet werden, um die einzelnen Sonden zu markieren,
iii) jeder Abschnitt mit einem anderen Emitterfluorophor markiert ist, und wobei Moleküle eines Abschnitts mit dem gleichen, einzigen Emitterfluorophor markiert sind,
iv) die Abschnitte gemischt sind, um eine einzige, eindeutig markierte Sonde zu erzeugen, die spezifisch ist für eines von den Nukleinsäure-Targets, und
v) die Hybridisierung jedes Sondenabschnitts mit seinem entsprechenden Nukleinsäure-Target zu einer nachweisbaren Fluoreszenzsignaländerung führt, die für seine Markierung bezeichnend ist;

b) Stimulieren der vorhandenen Fluorophore mit der Probe; und
c) Nachweisen von Änderungen in Emissionssignalen von den Emitterfluorophoren, ohne die Nukleinsäure-Targets räumlich voneinander zu trennen.

2. Assay nach Anspruch 1, ferner das exponentielle

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Amplifizieren der Nukleinsäure-Targets umfassend, wobei Änderungen in Emissionssignalen während der Amplifizierung optional in Echtzeit erkannt werden.

3. Assay nach Anspruch 2, wobei jede Sonde zwei, drei oder vier unterschiedlich markierte Abschnitte umfasst.

4. Assay nach einem der Ansprüche 1 bis 3, wobei die relativen Mengen der Abschnitte jeder Sonde in Bezug aufeinander angepasst sind, um ausgewogene Signaländerungen zu ermöglichen.

5. Assay nach einem der Ansprüche 1 bis 4, wobei der Satz von Sonden außerdem mindestens eine Sonde umfasst, die verhältniscodiert ist.

6. Assay nach einem der Ansprüche 1 bis 5, wobei die kombinatorisch codierten Sonden gequenchte Sonden sind, deren Fluoreszenz durch Hybridisierung an ihre Targets wiederhergestellt wird, wobei die kombinatorisch codierten Sonden optional Molecular-Beacon-Sonden sind, die einen nicht-fluoreszierenden Quencher an einem Zweig und einen Emitterfluorophor am anderen Zweig aufweisen, und wobei die Fluoreszenzintensitäten der Abschnitte jeder Sonde in Bezug aufeinander vorzugsweise ausgeglichen sind.

7. Assay nach Anspruch 6, ferner das exponentielle Amplifizieren der Nukleinsäure-Targets in Anwesenheit der Sonden und das Nachweisen von Zunahmen der Fluoreszenz von Emitterfluorophoren in Echtzeit umfassend.

8. Assay nach Anspruch 7, wobei jede Sonde zwei, drei oder vier verschieden markierte Abschnitte aus einem Panel von vier bis acht unterscheidbaren Fluorophoren umfasst, wobei die relativen Mengen jeder Sonde in Bezug aufeinander angepasst sind, um ausgewogene Fluoreszenzemissionen zu ermöglichen, wobei die relativen Mengen aller Abschnitte aller Sonden in Bezug aufeinander optional angepasst sind, um ausgewogene Fluoreszenzemissionen zu ermöglichen.

9. Kit von Reagenzien zur Verwendung in einem Mehrfach-*in-vitro*-Assay zur Durchmusterung einer Probe auf die Anwesenheit von mindestens sechs verschiedenen Nukleinsäure-Targets, einen Satz von mindestens sechs kombinatorisch codierten fluoreszierenden Hybridisierungssonden umfassend, wobei jede Sonde spezifisch ist für ein anderes Nukleinsäure-Target, wobei

i) jede Sonde in dem Satz mit einem kombinatorischen Code markiert ist, der mindestens zwei verschiedene Emitterfluorophore umfasst, die ausgewählt sind aus einem Panel von mindestens vier spektral unterschiedlichen Fluorophoren,

ii) jede targetspezifische Sonde in Abschnitte unterteilt ist, wobei jeder Abschnitt eine Population von Molekülen umfasst, und wobei die Anzahl der Abschnitte gleich der Anzahl von Emitterfluorophoren ist, die verwendet werden, um die einzelnen Sonden zu markieren,

iii) jeder Abschnitt mit einem anderen Emitterfluorophor markiert ist, und wobei Moleküle eines Abschnitts mit dem gleichen, einzigen Emitterfluorophor markiert sind,

iv) die Abschnitte gemischt sind, um eine einzige, eindeutig markierte Sonde zu erzeugen, die spezifisch ist für eines von den Nukleinsäure-Targets, und

v) die Hybridisierung jedes Sondenabschnitts mit seinem entsprechenden Nukleinsäure-Target zu einer nachweisbaren Fluoreszenzsignaländerung führt, die für seine Markierung bezeichnend ist;

10. Kit nach Anspruch 9, ferner umfassend:

(a) Primer für eine exponentielle Amplifizierung der Nukleinsäure-Targets,
(b) Amplifikationsreagenzien zum Amplifizieren der Nukleinsäure-Targets unter Verwendung der Primer; oder
(c) mindestens eine Sonde, die verhältniscodiert ist.

11. Kit nach Anspruch 9 oder Anspruch 10, wobei die kombinatorisch codierten Sonden gequenchte Sonden sind, deren Fluoreszenz durch Hybridisierung an ihre Targets wiederhergestellt wird, wobei die Sonden optional Molecular-Beacon-Sonden sind, die einen nicht-fluoreszierenden Quencher an einem Zweig und einen Emitterfluorophor am anderen Zweig aufweisen.

12. Kit nach Anspruch 11, wobei die relativen Mengen der Abschnitte jeder Sonde in Bezug aufeinander angepasst sind, um ausgewogene Fluoreszenzemissionen zu ermöglichen, wobei die relativen Mengen aller Abschnitte aller Sonden in Bezug aufeinander optional angepasst sind, um ausgewogene Fluoreszenzemissionen zu ermöglichen.

Revendications

1. Essai *in vitro* homogène multiplexe servant à balayer un échantillon pour détecter la présence d'au moins six cibles d'acide nucléique différentes, comprenant :

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a) la mise en contact de l'échantillon avec au moins six échantillons d'hybridation fluorescent à codage combinatoire, chaque échantillon étant spécifique à une cible d'acide nucléique différente, dans lequel

i) chaque échantillon dudit ensemble est étiqueté avec un code combinatoire comprenant au moins deux fluorophores émetteurs différents sélectionnés dans un groupe d'au moins quatre fluorophores différenciables au niveau spectral,
ii) chaque échantillon spécifique à une cible est subdivisé en sections, chaque section comprenant un peuplement de molécules, et dans lequel le nombre de sections est égal au nombre de fluorophores émetteurs utilisés pour étiqueter chaque échantillon,
iii) chaque section est étiquetée avec un fluorophore émetteur différent et dans lequel les molécules d'une section sont étiquetées avec le même unique fluorophore émetteur,
iv) lesdites sections sont mélangées pour créer un seul échantillon à étiquetage unique spécifique à une desdites cibles d'acide nucléique ; et
v) l'hybridation de chaque section d'échantillon avec sa cible d'acide nucléique correspondante entraîne un changement de signal fluorescent détectable indiquant son étiquetage ;

b) la stimulation des fluorophores présents avec l'échantillon ; et
c) la détection de changements dans des signaux d'émissions desdits émetteurs fluorophores sans isoler spatialement les cibles d'acide nucléique les unes des autres.

2. Essai selon la revendication1, comprenant en outre l'amplification exponentielle desdites cibles d'acide nucléique, dans lequel, en option, les changements des signaux d'émissions sont détectés en temps réel pendant l'amplification.

3. Essai selon la revendication 2, dans lequel chaque échantillon comprend deux, trois ou quatre sections étiquetées différemment.

4. Essai selon l'une quelconque des revendications 1 à 3, dans lequel les quantités relatives des sections de chaque échantillon sont ajustées les unes par rapport aux autres pour permettre des changements de signaux équilibrés.

5. Essai selon l'une quelconque des revendications 1 à 4, dans lequel ledit ensemble d'échantillons inclut au moins un échantillon qui est codé par ratio.

6. Essai selon l'une quelconque des revendications 1 à 5, dans lequel lesdits échantillons à codage combinatoire sont des échantillons trempés dont la fluorescence est restaurée par hybridation vers leurs cibles, dans lequel, en option, lesdits échantillons à codage combinatoire sont des échantillons à balise moléculaire dotés d'une balise non fluorescente sur un bras et d'un fluorophore émetteur sur l'autre bras et les intensités de fluorescence des sections de chaque échantillon sont de préférence équilibrées entre elles.

7. Essai selon la revendication 6, comprenant en outre l'amplification exponentielle desdites cibles d'acide nucléique en présence desdits échantillons et la détection d'augmentations de fluorescence provenant des fluorophores émetteurs en temps réel.

8. Essai selon la revendication 7, dans lequel chaque échantillon comprend deux, trois ou quatre fluorophores étiquetés différemment sur un groupe de quatre à huit fluorophores différenciables, dans lequel les quantités relatives de chaque échantillon sont ajustées les unes par rapport aux autres, dans lequel, en option, les quantités relatives de toutes les sections de tous les échantillons sont ajustées les unes par rapport aux autres pour permettre des émissions à fluorescence équilibrée.

9. Ensemble de réactifs destinés à un usage dans un essai *in vitro* homogène multiplexe servant à balayer un échantillon pour détecter la présence d'au moins six cibles d'acide nucléique différentes, comprenant un ensemble d'au moins six échantillons d'hybridation fluorescents à codage combinatoire, chaque échantillon étant spécifique à une cible d'acide nucléique différente, dans lequel

i) chaque échantillon dudit ensemble est étiqueté avec un code combinatoire comprenant au moins deux fluorophores émetteurs sélectionnés dans un groupe d'au moins quatre fluorophores différenciables au niveau spectral,
ii) chaque échantillon spécifique à une cible est subdivisé en sections, chaque section comprenant un peuplement de molécules, et dans lequel le nombre de sections est égal au nombre de fluorophores émetteurs utilisés pour étiqueter chaque échantillon,
iii) chaque section est étiquetée avec un fluorophore émetteur différent et dans lequel les molécules d'une section sont étiquetées avec le même unique fluorophore émetteur,
iv) lesdites sections sont mélangées pour créer un seul échantillon à étiquetage unique spécifique à une desdites cibles d'acide nucléique ; et

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v) l'hybridation de chaque section d'échantillon avec sa cible d'acide nucléique correspondante entraîne un changement de signal fluorescent détectable indiquant son étiquetage.

10. Ensemble selon la revendication 9, comprenant en outre :

(a) des primaires pour l'amplification exponentielle desdites cibles d'acide nucléique ;
(b) des réactifs d'amplification pour amplifier lesdites cibles d'acide nucléique en utilisant lesdits primaires ;
(c) au moins un échantillon qui est codé par ratio.

11. Ensemble selon la revendication 9 ou la revendication 10, dans lequel les échantillons à codage combine sont des échantillons trempés dont la fluorescence est restaurée par hybridation vers leurs cibles, dans lequel, en option, lesdits échantillons à codage combiné sont des échantillons à balise moléculaire dotés d'une balise fluorescente sur un bras et d'un fluorophore émetteur sur l'autre bras.

12. Ensemble selon la revendication 11, dans lequel les quantités relatives des sections de chaque échantillon sont ajustées les unes par rapport aux autres pour permettre des émissions à fluorescence équilibrée, dans lequel, en option, les quantités relatives de toutes les sections de tous les échantillons sont ajustées les unes par rapport aux autres pour permettre des émissions à fluorescence équilibrée.

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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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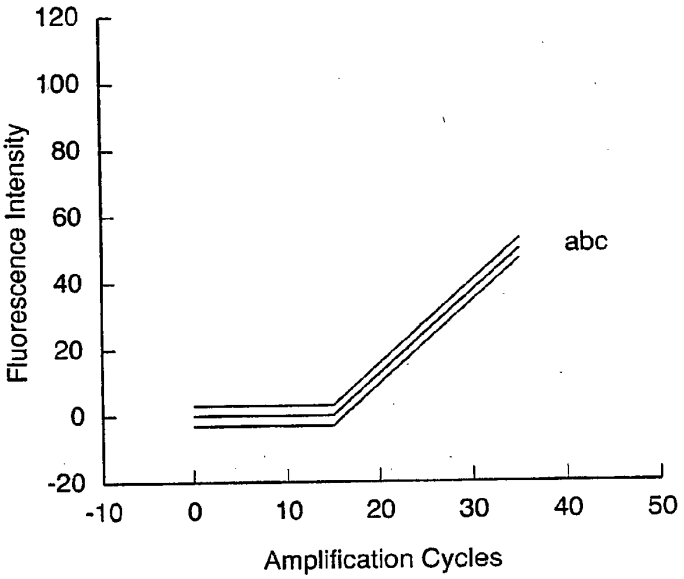


FIGURE 2

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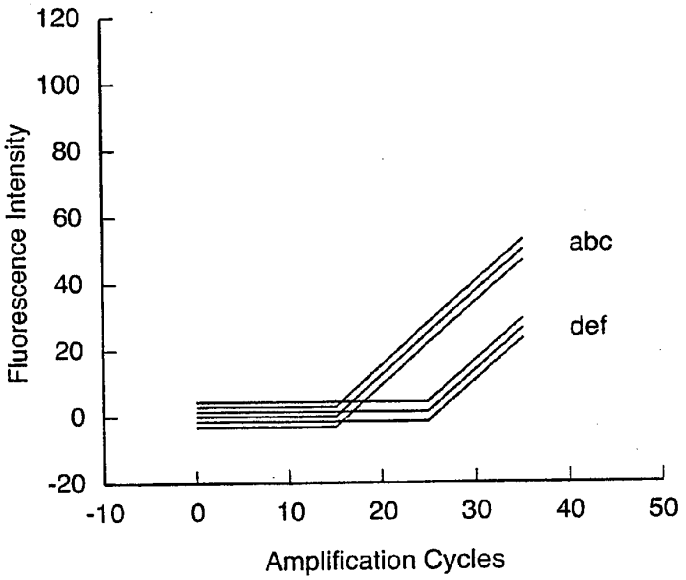


FIGURE 3

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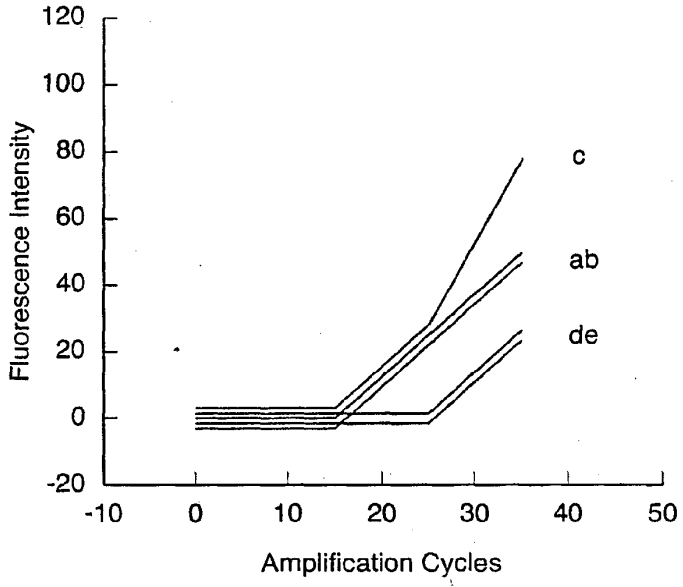


FIGURE 4

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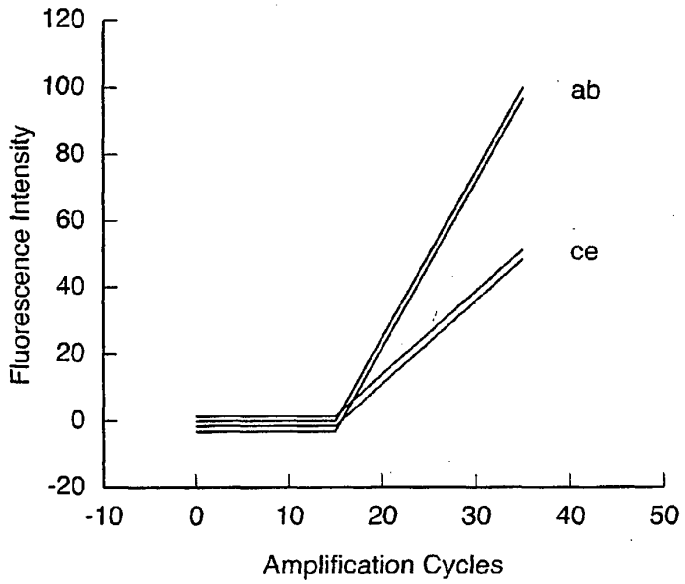


FIGURE 5

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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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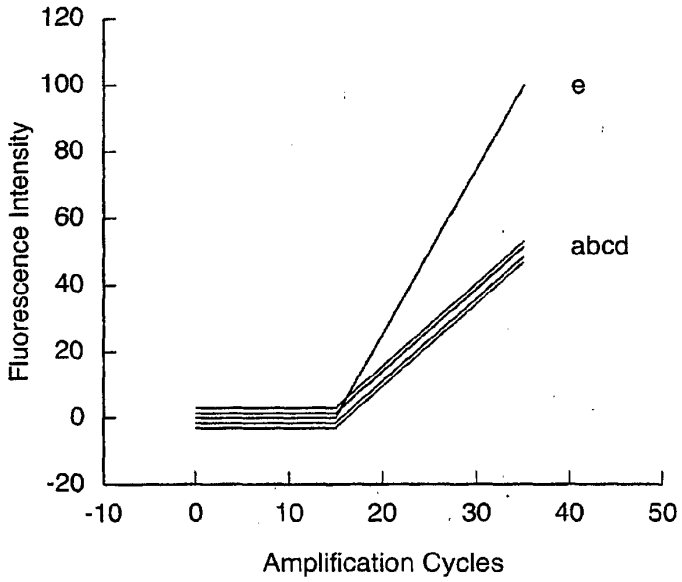


FIGURE 7

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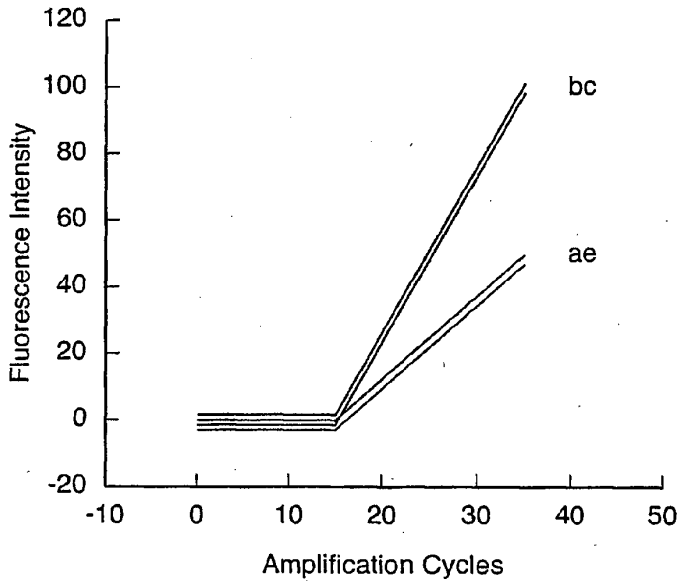


FIGURE 8

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REFERENCES CITED IN THE DESCRIPTION

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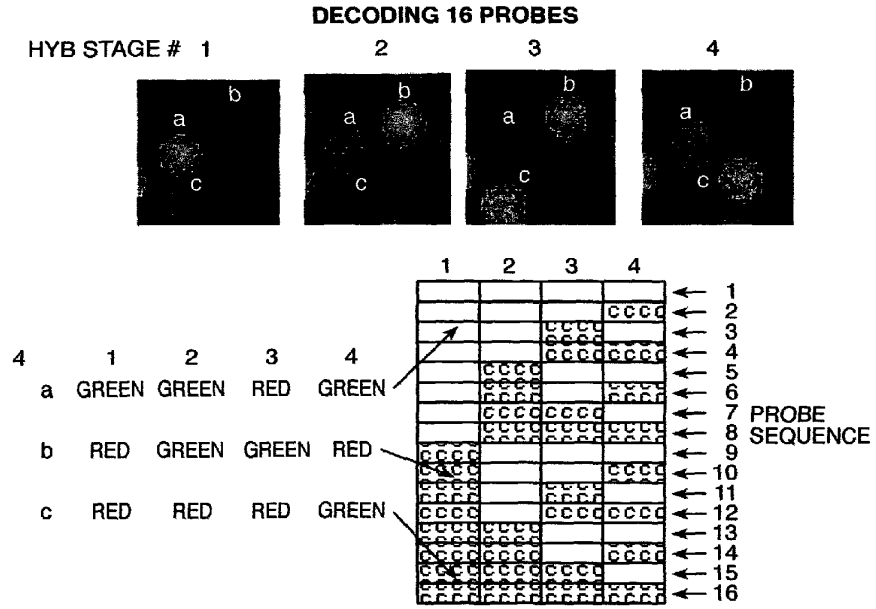
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[Continued on next page]

(54) Title: MULTIPLEX DECODING OF ARRAY SENSORS WITH MICROSPHERES



(57) Abstract: The invention relates to compositions and methods for multiplex decoding of microsphere array sensors.

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MULTIPLEX DECODING OF ARRAY SENSORS WITH MICROSPHERES

This application claims the benefit of U.S.S.N. 60/302, 213, filed June 28, 2001.

FIELD OF THE INVENTION

The invention relates to compositions and methods for decoding microsphere array sensors.

BACKGROUND OF THE INVENTION

There are a number of assays and sensors for the detection of the presence and/or concentration of specific substances in fluids and gases. Many of these rely on specific ligand/antiligand reactions as the mechanism of detection. That is, pairs of substances (i.e. the binding pairs or ligand/antiligands) are known to bind to each other, while binding little or not at all to other substances. This has been the focus of a number of techniques that utilize these binding pairs for the detection of the complexes. These generally are done by labeling one component of the complex in some way, so as to make the entire complex detectable, using, for example, radioisotopes, fluorescent and other optically active molecules, enzymes, etc.

Of particular use in these sensors are detection mechanisms utilizing luminescence. Recently, the use of optical fibers and optical fiber strands in combination with light absorbing dyes for chemical analytical determinations has undergone rapid development, particularly within the last decade. The use of optical fibers for such purposes and techniques is described by Milanovich et al., "Novel Optical Fiber Techniques For Medical Application", Proceedings of the SPIE 28th Annual International Technical Symposium On Optics and Electro-Optics, Volume 494, 1980; Seitz, W.R., "Chemical

Sensors Based On Immobilized Indicators and Fiber Optics" in *C.R.C. Critical Reviews In Analytical Chemistry*, Vol. 19, 1988, pp. 135-173; Wolfbeis, O.S., "Fiber Optical Fluorosensors In Analytical Chemistry" in *Molecular Luminescence Spectroscopy, Methods and Applications* (S. G. Schulman, editor), Wiley & Sons, New York (1988); Angel, S.M., *Spectroscopy 2* (4):38 (1987); Walt, et al., "Chemical Sensors and Microinstrumentation", *ACS Symposium Series*, Vol. 403, 1989, p. 252, and Wolfbeis, O.S., *Fiber Optic Chemical Sensors*, Ed. CRC Press, Boca Raton, FL, 1991, 2nd Volume.

When using an optical fiber in an *in vitro/in vivo* sensor, one or more light absorbing dyes are located near its distal end. Typically, light from an appropriate source is used to illuminate the dyes through the fiber's proximal end. The light propagates along the length of the optical fiber; and a portion of this propagated light exits the distal end and is absorbed by the dyes. The light absorbing dye may or may not be immobilized; may or may not be directly attached to the optical fiber itself; may or may not be suspended in a fluid sample containing one or more analytes of interest; and may or may not be retainable for subsequent use in a second optical determination.

Once the light has been absorbed by the dye, some light of varying wavelength and intensity returns, conveyed through either the same fiber or collection fiber(s) to a detection system where it is observed and measured. The interactions between the light conveyed by the optical fiber and the properties of the light absorbing dye provide an optical basis for both qualitative and quantitative determinations.

Of the many different classes of light absorbing dyes which conventionally are employed with bundles of fiber strands and optical fibers for different analytical purposes are those more common compositions that emit light after absorption termed "fluorophores" and those which absorb light and internally convert the absorbed light to heat, rather than emit it as light, termed "chromophores."

Fluorescence is a physical phenomenon based upon the ability of some molecules to absorb light (photons) at specified wavelengths and then emit light of a longer wavelength and at a lower energy. Substances able to fluoresce share a number of common characteristics: the ability to absorb light energy at one wavelength $_{ab}$; reach an excited energy state; and subsequently emit light at another light wavelength, $_{em}$. The absorption and fluorescence emission spectra are individual for each fluorophore and are often graphically represented as two separate curves that are slightly overlapping. The same fluorescence emission spectrum is generally observed irrespective of the wavelength of the exciting light and, accordingly, the wavelength and energy of the exciting light may be varied within limits; but the light emitted by the fluorophore will always provide the same emission spectrum. Finally, the strength of the fluorescence signal may be measured as the quantum yield of light emitted. The fluorescence quantum yield is the ratio of the number of photons emitted in comparison to the number of photons initially absorbed by the fluorophore. For more detailed information regarding each of these characteristics, the following references are recommended: Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*, Plenum Press, New York, 1983; Freifelder, D., Physical Biochemistry, second edition, W. H. Freeman and Company, New York, 1982; "Molecular Luminescence

Spectroscopy Methods and Applications: Part I" (S.G. Schulman, editor) in *Chemical Analysis*, vol. 77, Wiley & Sons, Inc., 1985; *The Theory of Luminescence*, Stepanov and Gribkovskii, Iliffe Books, Ltd., London, 1968.

In comparison, substances which absorb light and do not fluoresce usually convert the light into heat or kinetic energy. The ability to internally convert the absorbed light identifies the dye as a "chromophore." Dyes which absorb light energy as chromophores do so at individual wavelengths of energy and are characterized by a distinctive molar absorption coefficient at that wavelength. Chemical analysis employing fiber optic strands and absorption spectroscopy using visible and ultraviolet light wavelengths in combination with the absorption coefficient allow for the determination of concentration for specific analyses of interest by spectral measurement. The most common use of absorbance measurement via optical fibers is to determine concentration which is calculated in accordance with Beers' law; accordingly, at a single absorbance wavelength, the greater the quantity of the composition which absorbs light energy at a given wavelength, the greater the optical density for the sample. In this way, the total quantity of light absorbed directly correlates with the quantity of the composition in the sample.

Many of the recent improvements employing optical fiber sensors in both qualitative and quantitative analytical determinations concern the desirability of depositing and/or immobilizing various light absorbing dyes at the distal end of the optical fiber. In this manner, a variety of different optical fiber chemical sensors and methods have been reported for specific analytical determinations and applications such as pH measurement, oxygen detection, and carbon dioxide analyses. These developments are exemplified by the following publications: Freeman, et al., *Anal Chem.* 53:98 (1983); Lippitsch et al., *Anal. Chem. Acta.* 205:1, (1988); Wolfbeis et al., *Anal. Chem.* 60:2028 (1988); Jordan, et al., *Anal. Chem.* 59:437 (1987); Lubbers et al., *Sens. Actuators* 1983; Munkholm et al., *Talanta* 35:109 (1988); Munkholm et al., *Anal. Chem.* 58:1427 (1986); Seitz, W. R., *Anal. Chem.* 56:16A-34A (1984); Peterson, et al., *Anal. Chem.* 52:864 (1980): Saari, et al., *Anal. Chem.* 54:821 (1982); Saari, et al., *Anal. Chem.* 55:667 (1983); Zhujun et al., *Anal. Chem. Acta.* 160:47 (1984); Schwab, et al., *Anal. Chem.* 56:2199 (1984); Wolfbeis, O.S., "Fiber Optic Chemical Sensors", *Ed. CRC Press, Boca Raton, FL*, 1991, 2nd Volume; and Pantano, P., Walt, D.R., *Anal. Chem.*, 481A-487A, Vol. 67, (1995).

More recently, fiber optic sensors have been constructed that permit the use of multiple dyes with a single, discrete fiber optic bundle. U.S. Pat. Nos. 5,244,636 and 5,250,264 to Walt, *et al.* disclose systems for affixing multiple, different dyes on the distal end of the bundle, the teachings of each of these patents being incorporated herein by this reference. The disclosed configurations enable separate optical fibers of the bundle to optically access individual dyes. This avoids the problem of deconvolving the separate signals in the returning light from each dye, which arises when the signals from two or more dyes are combined, each dye being sensitive to a different analyte, and there is significant overlap in the dyes' emission spectra.

U.S.S.N.s 08/818,199 and 09/151,877 describe array compositions that utilize microspheres or beads on a surface of a substrate, for example on a terminal end of a fiber optic bundle, with each individual fiber comprising a bead containing an optical signature. Since the beads go down randomly, a unique optical signature is needed to "decode" the array; i.e. after the array is made, a correlation of the location of an individual site on the array with the bead or bioactive agent at that particular site can be made. This means that the beads may be randomly distributed on the array, a fast and inexpensive process as compared to either the *in situ* synthesis or spotting techniques of the prior art. Once the array is loaded with the beads, the array can be decoded, or can be used, with full or partial decoding occurring after testing, as is more fully outlined below.

One drawback with the previous system is that it requires a set of unique optical signatures. While large sets of such signatures are available, for example by using different ratios of different dyes, it would be preferable to use decoding systems that do not rely on the use of sets of optical signatures. Accordingly, it is an object of the invention to provide methods to allow decoding of bead arrays without relying solely on unique optical signatures.

SUMMARY OF THE INVENTION

In accordance with the above objects, the present invention provides array compositions comprising a substrate with a surface comprising discrete sites. The composition further comprises a population of microspheres comprising at least a first and a second subpopulation; each subpopulation comprises a bioactive agent; and an identifier binding ligand that will bind a decoder binding ligand such that the identity of the bioactive agent can be elucidated. The microspheres are distributed on the surface.

In a further aspect, the present invention provides array compositions comprising a substrate with a surface comprising discrete sites, and a population of microspheres comprising at least a first and a second subpopulation. Each subpopulation comprises a bioactive agent and does not comprise an optical signature.

In an additional aspect, the present invention provides methods of making an array composition as outlined above. The methods comprise forming a surface comprising individual sites on a substrate and distributing microspheres on said surface such that said individual sites contain microspheres. The microspheres comprise at least a first and a second subpopulation each comprising a bioactive agent and do not comprise an optical signature.

In a further aspect, the invention provides methods of making a composition comprising forming a surface comprising individual sites on a substrate and distributing microspheres on the surface such that the individual sites contain microspheres. The microspheres comprise at least a first and a second subpopulation each comprising a bioactive agent and an identifier binding ligand that will bind a decoder binding ligand such that the identification of the bioactive agent can be elucidated.

In an additional aspect, the invention provides methods of decoding an array composition comprising providing an array composition as outlined above, and adding a plurality of decoding binding ligands to the array composition to identify the location of at least a plurality of the bioactive agents.

In a further aspect, the invention provides methods of determining the presence of a target analyte in a sample. The methods comprise contacting the sample with an array composition as outlined herein, and determining the presence or absence of the target analyte.

In a further aspect, the invention provides a method comprising providing an array composition comprising a population of microspheres comprising at least a first and a second subpopulation, wherein each subpopulation comprises a bioactive agent and at least a first and a second decoding attribute, and detecting each of said first and second decoding attributes to identify each of said bioactive agents.

In a further aspect the invention provides a method of increasing the information obtained in a decoding step. The method includes the use of degenerate probes as DBL-IBL combinations. In addition the invention provides the use of multiple decoding attributes on a bead.

In further aspect, the invention provides a method of increasing the confidence of decoding. The method includes using the decoding as a quality control measure. In addition, the invention provides quality control fibers. In addition, the invention provides for parity analysis of decoding data.

In a further aspect, the invention provides a method of decoding an array composition comprising providing an array composition comprising a population of microspheres comprising at least 50 subpopulations, wherein each subpopulation comprises a bioactive agent adding a plurality of decoding binding ligands to said population of microspheres to identify at least 50 of the bioactive agents.

In a further aspect, the invention provides a method of determining the presence of a target analyte in a sample comprising contacting said sample with a composition comprising a population of microspheres comprising at least 50 subpopulations, wherein each subpopulation comprises a bioactive agent adding a plurality of decoding binding ligands to said population of microspheres to identify at least 50 of the bioactive agents and determining the presence or absence of said target analyte.

FIGURES

Fig. 1 Illustrates a two color decoding process wherein either FAM-labeled or Cy3-labeled oligo complements are use to "paint" (label) the different bead types on the array.

Fig. 2 Depicts the decoding 128 different bead types with four colors and four decode stages. (inset shows a single decode stage using four different dyes to decode 16 bead types.)

Fig. 3. Grey scale decoding of 16 different bead types. (A) Combinatorial pooling scheme for complementary decoding oligos. A (B) Two independent normalizing images were acquired, and the resulting bead intensities compared. (C) The alpha values (ratio of bead intensity in indicated decode stage to intensity in normalization image) are plotted for three decodes stage described in (A).

Fig. 4 Depicts the use of fluorescence resonance energy transfer as a code for a probe on a random array. A. Depicts FRET oligos with linkers of different lengths. B. Depicts a plot of the ration of emission of the fluors from the oligos in Fig. 4A.

Figure 5 Use of fluorescence resonance energy transfer to discriminate between linkers of varying length.

Figure 6 depicts clustering in hyperspectral alpha space ($\Delta_1 = I_1/6I_I$, $\Delta_2 = I_2/6I_i$, $\Delta_3 = I_3/6I_i$, etc.). A set of 128 different bead types present on a fiber bundle were decoded with by hybridizing set of complementary oligonucleotides labeled with four dyes: Bodipy-493, Bodipy-R6G, Bodipy-TXR, and Bod-564 (only one dye per oligonucleotide). Shown is the second stage of a four stage decode in which 4013 beads were decoded. Ovals are drawn around zones of hue clusters.

Figure 7 depicts an error checking scheme.

Figure 8 depicts an example of primer extension labeling of decoder oligonucleotides.

Figure 9 depicts two color decoding of 16 beads with 16 distinct IBLs.

Figure 10 depicts primer extension labeling of degenerate IBL-DBL probes.

Figure 11 depicts a scatter plot of signals obtained from three distinct labels recorded in two channels.

Figure 12 depicts decoding by sequencing. A polymerase (e.g. Sequenase, Thermosequenase) and dye-labeled chain-terminating nucleotides are used to sequence the encoding region of a DNA strand present on the bead. The four nucleotides are each labeled with a discrete label. Because only 4 colors are detected at once, the colors are removed after each extension step. In subsequent extension steps, the primers are indexed or extended by one base so that the primer can query the next base in the encoding region.

Figure 13 Depicts construction of probes on bead containing encoding sequences, zipcodes, and a gene-specific sequence. Two different encoding cassettes are employed to facilitate the primer extension reaction using primers with universal or degenerate bases. Using four color sequencing and a single base code, 6 bases generates 4^6 = 4096 codes, likewise four color hybridization using single hybridization colors also generates 4096 codes. The grand total number of codes is 4096*4096 = >16 million. If only a single color/two state scheme is employed for hybridization, than 2^6 = 64 codes are generated. The grand total is 4096*64 = 262,144 codes. The zipcode sequences can also be constructed so as to be overlapping to reduce the length of the overall sequence (i.e. cZip#1 vs. cZip#2).

Figure 14 Depicts incorporation of a cleavable linker between encoding sequences and gene-specific sequence. A disulfide cleavable linker is shown for illustration purposes. If the linker is not present, the terminal portion of the gene-specific sequence can be included as part of the encoding sequence to reduce overall length of the probe.

Figure 15 depicts decoding with exonuclease.

Figure 16 depicts a graph showing decoding of about 1500 bead subpopulation on each of several different fiber bundles.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is generally based on previous work comprising a bead-based analytic chemistry system in which beads, also termed microspheres, carrying different chemical functionalities are distributed on a substrate comprising a patterned surface of discrete sites that can bind the individual microspheres. Since the beads are generally put onto the substrate randomly, the previous work relied on the incorporation of unique optical signatures, generally fluorescent dyes, that could be used to identify the chemical functionality on any particular bead. This allows the synthesis of the candidate agents (i.e. compounds such as nucleic acids and antibodies) to be divorced from their placement on an array, i.e. the candidate agents may be synthesized on the beads, and then the beads are randomly distributed on a patterned surface. Since the beads are first coded with an optical signature, this means that the array can later be "decoded", i.e. after the array is made, a correlation of the location of an individual site on the array with the bead or candidate agent at that particular site can be made. This means that the beads may be randomly distributed on the array, a fast and inexpensive process as compared to either the in situ synthesis or spotting techniques of the prior art.

However, the drawback to these methods is that for a large array, the system requires a large number of different optical signatures, which may be difficult or time-consuming to utilize. Accordingly, the present invention provides several improvements over these methods, generally directed to methods of coding and decoding the arrays. That is, as will be appreciated by those in the art, the placement of

the bioactive agents is generally random, and thus a coding/decoding system is required to identify the bioactive agent at each location in the array. This may be done in a variety of ways, as is more fully outlined below, and generally includes: a) the use of decoding binding ligands (DBLs), generally directly labeled, that binds to either the bioactive agent or to identifier binding ligands (IBLs) attached to the beads; b) positional decoding, for example by either targeting the placement of beads (for example by using photoactivatible or photocleavable moieties to allow the selective addition of beads to particular locations), or by using either sub-bundles or selective loading of the sites, as are more fully outlined below; c) selective decoding, wherein only those beads that bind to a target are decoded; or d) combinations of any of these. In some cases, as is more fully outlined below, this decoding may occur for all the beads, or only for those that bind a particular target analyte. Similarly, this may occur either prior to or after addition of a target analyte.

Once the identity of the bioactive agent and its location in the array has been fixed, the array is exposed to samples containing the target analytes, although as outlined below, this can be done prior to or during the analysis as well. The target analytes will bind to the bioactive agents as is more fully outlined below, and result in a change in the optical signal of a particular bead.

In the present invention, "decoding" does not rely on the use of optical signatures, but rather on the use of decoding binding ligands that are added during a decoding step. The decoding binding ligands will bind either to a distinct identifier binding ligand partner that is placed on the beads, or to the bioactive agent itself, for example when the beads comprise single-stranded nucleic acids as the bioactive agents. The decoding binding ligands are either directly or indirectly labeled, and thus decoding occurs by detecting the presence of the label. By using pools of decoding binding ligands in a sequential fashion, it is possible to greatly minimize the number of required decoding steps.

In one embodiment, the invention provides methods for multiplex decoding of bead-arrays. That is, microspheres with bioactive agents are decoded to elucidate the identity of the bioactive agent on the bead. In a preferred embodiment at least 50 different bead types or subpopulations are simultaneously decoded, or decoded in a single decoding experiment. More preferably at least 100; more preferably at least 500; more preferably at least 1000; or preferably at least 1500 bead types are simultaneously decoded or decoded in a single decoding experiment by the methods of the invention. Preferably at least 5000 bead types are simultaneously decoded or decoded in a single decoding experiment.

Accordingly, the present invention provides array compositions comprising at least a first substrate with a surface comprising individual sites. By "array" herein is meant a plurality of candidate agents in an array format; the size of the array will depend on the composition and end use of the array. Arrays containing from about 2 different bioactive agents (i.e. different beads) to many millions can be made, with very large fiber optic arrays being possible. Generally, the array will comprise from two to as many as a billion or more, depending on the size of the beads and the substrate, as well as the end use of the array, thus very high density, high density, moderate density, low density and very low

density arrays may be made. Preferred ranges for very high density arrays are from about 10,000,000 to about 2,000,000,000 (all numbers are per square cm), with from about 100,000,000 to about 1,000,000,000 being preferred. High density arrays range about 100,000 to about 10,000,000, with from about 1,000,000 to about 5,000,000 being particularly preferred. Moderate density arrays range from about 10,000 to about 100,000 being particularly preferred, and from about 20,000 to about 50,000 being especially preferred. Low density arrays are generally less than 10,000, with from about 1,000 to about 5,000 being preferred. Very low density arrays are less than 1,000, with from about 10 to about 1000 being preferred, and from about 100 to about 500 being particularly preferred. In some embodiments, the compositions of the invention may not be in array format; that is, for some embodiments, compositions comprising a single bioactive agent may be made as well. In addition, in some arrays, multiple substrates may be used, either of different or identical compositions. Thus for example, large arrays may comprise a plurality of smaller substrates.

In addition, one advantage of the present compositions is that particularly through the use of fiber optic technology, extremely high density arrays can be made. Thus for example, because beads of 200 m or less (with beads of 200 nm possible) can be used, and very small fibers are known, it is possible to have as many as 40,000 or more (in some instances, 1 million) different fibers and beads in a 1 mm^2 fiber optic bundle, with densities of greater than 15,000,000 individual beads and fibers (again, in some instances as many as 25-50 million) per 0.5 cm^2 obtainable.

By "substrate" or "solid support" or other grammatical equivalents herein is meant any material that can be modified to contain discrete individual sites appropriate for the attachment or association of beads and is amenable to at least one detection method. As will be appreciated by those in the art, the number of possible substrates is very large. Possible substrates include, but are not limited to, glass and modified or functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, Teflon, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, optical fiber bundles, and a variety of other polymers. In general, the substrates allow optical detection and do not themselves appreciably fluoresce.

Generally the substrate is flat (planar), although as will be appreciated by those in the art, other configurations of substrates may be used as well; for example, three dimensional configurations can be used, for example by embedding the beads in a porous block of plastic that allows sample access to the beads and using a confocal microscope for detection. Similarly, the beads may be placed on the inside surface of a tube, for flow-through sample analysis to minimize sample volume. Preferred substrates include optical fiber bundles as discussed below, and flat planar substrates such as glass, polystyrene and other plastics and acrylics.

In a preferred embodiment, the substrate is an optical fiber bundle or array, as is generally described in U.S.S.N.s 08/944,850 and 08/519,062, PCT US98/05025, and PCT US98/09163, all of which are expressly incorporated herein by reference. Preferred embodiments utilize preformed unitary fiber optic arrays. By "preformed unitary fiber optic array" herein is meant an array of discrete individual fiber optic strands that are co-axially disposed and joined along their lengths. The fiber strands are generally individually clad. However, one thing that distinguished a preformed unitary array from other fiber optic formats is that the fibers are not individually physically manipulatable; that is, one strand generally cannot be physically separated at any point along its length from another fiber strand.

Generally, the array of array compositions of the invention can be configured in several ways; see for example U.S.S.N. 09/473,904, and WO 00/39587, both of which are hereby expressly incorporated by reference. In a preferred embodiment, as is more fully outlined below, a "one component" system is used. That is, a first substrate comprising a plurality of assay locations (sometimes also referred to herein as "assay wells"), such as a microtiter plate, is configured such that each assay location contains an individual array. That is, the assay location and the array location are the same. For example, the plastic material of the microtiter plate can be formed to contain a plurality of "bead wells" in the bottom of each of the assay wells. Beads containing the capture probes of the invention can then be loaded into the bead wells in each assay location as is more fully described below.

Alternatively, a "two component" system can be used. In this embodiment, the individual arrays are formed on a second substrate, which then can be fitted or "dipped" into the first microtiter plate substrate. A preferred embodiment utilizes fiber optic bundles as the individual arrays, generally with "bead wells" etched into one surface of each individual fiber, such that the beads containing the capture probes are loaded onto the end of the fiber optic bundle. The composite array thus comprises a number of individual arrays that are configured to fit within the wells of a microtiter plate.

By "composite array" or "combination array" or grammatical equivalents herein is meant a plurality of individual arrays, as outlined above. Generally the number of individual arrays is set by the size of the microtiter plate used; thus, 96 well, 384 well and 1536 well microtiter plates utilize composite arrays comprising 96, 384 and 1536 individual arrays, although as will be appreciated by those in the art, not each microtiter well need contain an individual array. It should be noted that the composite arrays can comprise individual arrays that are identical, similar or different. That is, in some embodiments, it may be desirable to do the same 2,000 assays on 96 different samples; alternatively, doing 192,000 experiments on the same sample (i.e. the same sample in each of the 96 wells) may be desirable. Alternatively, each row or column of the composite array could be the same, for redundancy/quality control. As will be appreciated by those in the art, there are a variety of ways to configure the system. In addition, the random nature of the arrays may mean that the same population of beads may be added to two different surfaces, resulting in substantially similar but perhaps not identical arrays.

At least one surface of the substrate is modified to contain discrete, individual sites for later association of microspheres. These sites may comprise physically altered sites, i.e. physical configurations such as wells or small depressions in the substrate that can retain the beads, such that a microsphere can rest in the well, or the use of other forces (magnetic or compressive), or chemically altered or active sites, such as chemically functionalized sites, electrostatically altered sites, hydrophobically/ hydrophilically functionalized sites, spots of adhesive, etc.

The sites may be a pattern, i.e. a regular design or configuration, or randomly distributed. A preferred embodiment utilizes a regular pattern of sites such that the sites may be addressed in the X-Y coordinate plane. "Pattern" in this sense includes a repeating unit cell, preferably one that allows a high density of beads on the substrate. However, it should be noted that these sites may not be discrete sites. That is, it is possible to use a uniform surface of adhesive or chemical functionalities, for example, that allows the association of beads at any position. That is, the surface of the substrate is modified to allow association of the microspheres at individual sites, whether or not those sites are contiguous or non-contiguous with other sites. Thus, the surface of the substrate may be modified such that discrete sites are formed that can only have a single associated bead, or alternatively, the surface of the substrate is modified and beads may go down anywhere, but they end up at discrete sites.

In a preferred embodiment, the surface of the substrate is modified to contain wells, i.e. depressions in the surface of the substrate. This may be done as is generally known in the art using a variety of techniques, including, but not limited to, photolithography, stamping techniques, molding techniques and microetching techniques. As will be appreciated by those in the art, the technique used will depend on the composition and shape of the substrate.

In a preferred embodiment, physical alterations are made in a surface of the substrate to produce the sites. In a preferred embodiment, the substrate is a fiber optic bundle and the surface of the substrate is a terminal end of the fiber bundle, as is generally described in 08/818,199 and 09/151,877, both of which are hereby expressly incorporated by reference. In this embodiment, wells are made in a terminal or distal end of a fiber optic bundle comprising individual fibers. In this embodiment, the cores of the individual fibers are etched, with respect to the cladding, such that small wells or depressions are formed at one end of the fibers. The required depth of the wells will depend on the size of the beads to be added to the wells.

Generally in this embodiment, the microspheres are non-covalently associated in the wells, although the wells may additionally be chemically functionalized as is generally described below, cross-linking agents may be used, or a physical barrier may be used, i.e. a film or membrane over the beads.

In a preferred embodiment, the surface of the substrate is modified to contain chemically modified sites, that can be used to associate, either covalently or non-covalently, the microspheres of the invention to the discrete sites or locations on the substrate. "Chemically modified sites" in this context includes, but is not limited to, the addition of a pattern of chemical functional groups including amino groups, carboxy groups, oxo groups and thiol groups, that can be used to covalently attach microspheres, which generally also contain corresponding reactive functional groups; the addition of a pattern of adhesive that can be used to bind the microspheres (either by prior chemical functionalization for the addition of the adhesive or direct addition of the adhesive); the addition of a pattern of charged groups (similar to the chemical functionalities) for the electrostatic association of the microspheres, i.e. when the microspheres comprise charged groups opposite to the sites; the addition of a pattern of chemical functional groups that renders the sites differentially hydrophobic or hydrophilic, such that the addition of similarly hydrophobic or hydrophilic microspheres under suitable experimental conditions will result in association of the microspheres to the sites on the basis of hydroaffinity. For example, the use of hydrophobic sites with hydrophobic beads, in an aqueous system, drives the association of the beads preferentially onto the sites. As outlined above, "pattern" in this sense includes the use of a uniform treatment of the surface to allow association of the beads at discrete sites, as well as treatment of the surface resulting in discrete sites. As will be appreciated by those in the art, this may be accomplished in a variety of ways.

The compositions of the invention further comprise a population of microspheres. By "population" herein is meant a plurality of beads as outlined above for arrays. Within the population are separate subpopulations, which can be a single microsphere or multiple identical microspheres. That is, in some embodiments, as is more fully outlined below, the array may contain only a single bead for each bioactive agent; preferred embodiments utilize a plurality of beads of each type.

By "microspheres" or "beads" or "particles" or grammatical equivalents herein is meant small discrete particles. The composition of the beads will vary, depending on the class of bioactive agent and the method of synthesis. Suitable bead compositions include those used in peptide, nucleic acid and organic moiety synthesis, including, but not limited to, plastics, ceramics, glass, polystyrene, methylstyrene, acrylic polymers, paramagnetic materials, thoria sol, carbon graphite, titanium dioxide, latex or cross-linked dextrans such as Sepharose, cellulose, nylon, cross-linked micelles and Teflon may all be used. *"Microsphere Detection Guide"* from Bangs Laboratories, Fishers IN is a helpful guide.

The beads need not be spherical; irregular particles may be used. In addition, the beads may be porous, thus increasing the surface area of the bead available for either bioactive agent attachment or IBL attachment. The bead sizes range from nanometers, i.e. 100 nm, to millimeters, i.e. 1 mm, with

beads from about 0.2 micron to about 200 microns being preferred, and from about 0.5 to about 5 micron being particularly preferred, although in some embodiments smaller beads may be used.

It should be noted that a key component of the invention is the use of a substrate/bead pairing that allows the association or attachment of the beads at discrete sites on the surface of the substrate, such that the beads do not move during the course of the assay.

Each microsphere comprises a bioactive agent, although as will be appreciated by those in the art, there may be some microspheres which do not contain a bioactive agent, depending on the synthetic methods. By "candidate bioactive agent" or "bioactive agent" or "chemical functionality" or "binding ligand" herein is meant as used herein describes any molecule, *e.g.*, protein, oligopeptide, small organic molecule, coordination complex, polysaccharide, polynucleotide, etc. which can be attached to the microspheres of the invention. It should be understood that the compositions of the invention have two primary uses. In a preferred embodiment, as is more fully outlined below, the compositions are used to detect the presence of a particular target analyte; for example, the presence or absence of a particular nucleotide sequence or a particular protein, such as an enzyme, an antibody or an antigen. In an alternate preferred embodiment, the compositions are used to screen bioactive agents, i.e. drug candidates, for binding to a particular target analyte.

Bioactive agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 100 and less than about 2,500 Daltons. Bioactive agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The bioactive agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Bioactive agents are also found among biomolecules including peptides, nucleic acids, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Particularly preferred are nucleic acids and proteins.

Bioactive agents can be obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical

modifications, such as acylation, alkylation, esterification and/or amidification to produce structural analogs.

In a preferred embodiment, the bioactive agents are proteins. By "protein" herein is meant at least two covalently attached amino acids, which includes proteins, polypeptides, oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures. Thus "amino acid", or "peptide residue", as used herein means both naturally occurring and synthetic amino acids. For example, homo-phenylalanine, citrulline and norleucine are considered amino acids for the purposes of the invention. The side chains may be in either the (R) or the (S) configuration. In the preferred embodiment, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example to prevent or retard *in vivo* degradations.

In one preferred embodiment, the bioactive agents are naturally occurring proteins or fragments of naturally occuring proteins. Thus, for example, cellular extracts containing proteins, or random or directed digests of proteinaceous cellular extracts, may be used. In this way libraries of procaryotic and eukaryotic proteins may be made for screening in the systems described herein. Particularly preferred in this embodiment are libraries of bacterial, fungal, viral, and mammalian proteins, with the latter being preferred, and human proteins being especially preferred.

In a preferred embodiment, the bioactive agents are peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred, and from about 7 to about 15 being particularly preferred. The peptides may be digests of naturally occurring proteins as is outlined above, random peptides, or "biased" random peptides. By "randomized" or grammatical equivalents herein is meant that each nucleic acid and peptide consists of essentially random nucleotides and amino acids, respectively. Since generally these random peptides (or nucleic acids, discussed below) are chemically synthesized, they may incorporate any nucleotide or amino acid at any position. The synthetic process can be designed to generate randomized proteins or nucleic acids, to allow the formation of all or most of the possible combinations over the length of the sequence, thus forming a library of randomized bioactive proteinaceous agents.

In a preferred embodiment, a library of bioactive agents are used. The library should provide a sufficiently structurally diverse population of bioactive agents to effect a probabilistically sufficient range of binding to target analytes. Accordingly, an interaction library must be large enough so that at least one of its members will have a structure that gives it affinity for the target analyte. Although it is difficult to gauge the required absolute size of an interaction library, nature provides a hint with the immune response: a diversity of 10^7-10^8 different antibodies provides at least one combination with sufficient affinity to interact with most potential antigens faced by an organism. Published in vitro

selection techniques have also shown that a library size of 10^7 to 10^8 is sufficient to find structures with affinity for the target. Thus, in a preferred embodiment, at least 10^6, preferably at least 10^7, more preferably at least 10^8 and most preferably at least 10^9 different bioactive agents are simultaneously analyzed in the subject methods. Preferred methods maximize library size and diversity.

In a preferred embodiment, the library is fully randomized, with no sequence preferences or constants at any position. In a preferred embodiment, the library is biased. That is, some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, in a preferred embodiment, the nucleotides or amino acid residues are randomized within a defined class, for example, of hydrophobic amino acids, hydrophilic residues, sterically biased (either small or large) residues, towards the creation of cysteines, for cross-linking, prolines for SH-3 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, etc., or to purines, etc.

In a preferred embodiment, the bioactive agents are nucleic acids (generally called "probe nucleic acids" or "candidate probes" herein). By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramide (Beaucage, *et al.*, Tetrahedron, **49**(10):1925 (1993) and references therein; Letsinger, J. Org. Chem., **35**:3800 (1970); Sprinzl, *et al.*, Eur. J. Biochem., **81**:579 (1977); Letsinger, *et al.*, Nucl. Acids Res., **14**:3487 (1986); Sawai, *et al.*, Chem. Lett., 805 (1984), Letsinger, *et al.*, J. Am. Chem. Soc., **110**:4470 (1988); and Pauwels, *et al.*, Chemica Scripta, **26**:141 (1986)), phosphorothioate (Mag, *et al.*, Nucleic Acids Res., **19**:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu, *et al.*, J. Am. Chem. Soc., **111**:2321 (1989)), O-methylphophoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. Am. Chem. Soc., **114**:1895 (1992); Meier, *et al.*, Chem. Int. Ed. Engl., **31**:1008 (1992); Nielsen, Nature, **365**:566 (1993); Carlsson, *et al.*, Nature, **380**:207 (1996), all of which are incorporated by reference)). Other analog nucleic acids include those with positive backbones (Denpcy, *et al.*, Proc. Natl. Acad. Sci. USA, **92**:6097 (1995)); non-ionic backbones (U.S. Patent Nos. 5,386,023; 5,637,684; 5,602,240; 5,216,141; and 4,469,863; Kiedrowshi, *et al.*, Angew. Chem. Intl. Ed. English, **30**:423 (1991); Letsinger, *et al.*, J. Am. Chem. Soc., **110**:4470 (1988); Letsinger, *et al.*, Nucleosides & Nucleotides, **13**:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker, *et al.*, Bioorganic & Medicinal Chem. Lett., **4**:395 (1994); Jeffs, *et al.*, J. Biomolecular NMR, **34**:17 (1994); Tetrahedron Lett., **37**:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of

nucleic acids (see Jenkins, *et al.*, Chem. Soc. Rev., (1995) pp. 169-176). Several nucleic acid analogs are described in Rawls, C & E News, June 2, 1997, page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-life of such molecules in physiological environments; for example, PNA is particularly preferred. In addition, mixtures of naturally occurring nucleic acids and analogs can be made. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made. The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthanine, hypoxanthanine, isocytosine, isoguanine, and base analogs such as nitropyrrole and nitroindole, etc.

In a preferred embodiment, the bioactive agents are libraries of clonal nucleic acids, including DNA and RNA. In this embodiment, individual nucleic acids are prepared, generally using conventional methods (including, but not limited to, propagation in plasmid or phage vectors, amplification techniques including PCR, etc.). The nucleic acids are preferably arrayed in some format, such as a microtiter plate format, and beads added for attachment of the libraries.

Attachment of the clonal libraries (or any of the nucleic acids outlined herein) may be done in a variety of ways, as will be appreciated by those in the art, including, but not limited to, chemical or affinity capture (for example, including the incorporation of derivatized nucleotides such as AminoLink or biotinylated nucleotides that can then be used to attach the nucleic acid to a surface, as well as affinity capture by hybridization), cross-linking, and electrostatic attachment, etc.

In a preferred embodiment, affinity capture is used to attach the clonal nucleic acids to the beads. For example, cloned nucleic acids can be derivatized, for example with one member of a binding pair, and the beads derivatized with the other member of a binding pair. Suitable binding pairs are as described herein for IBL/DBL pairs. For example, the cloned nucleic acids may be biotinylated (for example using enzymatic incorporate of biotinylated nucleotides, for by photoactivated cross-linking of biotin). Biotinylated nucleic acids can then be captured on streptavidin-coated beads, as is known in the art. Similarly, other hapten-receptor combinations can be used, such as digoxigenin and anti-digoxigenin antibodies. Alternatively, chemical groups can be added in the form of derivatized nucleotides, that can them be used to add the nucleic acid to the surface.

Preferred attachments are covalent, although even relatively weak interactions (i.e. non-covalent) can be sufficient to attach a nucleic acid to a surface, if there are multiple sites of attachment per each

nucleic acid. Thus, for example, electrostatic interactions can be used for attachment, for example by having beads carrying the opposite charge to the bioactive agent.

Similarly, affinity capture utilizing hybridization can be used to attach cloned nucleic acids to beads. For example, as is known in the art, polyA+RNA is routinely captured by hybridization to oligo-dT beads; this may include oligo-dT capture followed by a cross-linking step, such as psoralen crosslinking). If the nucleic acids of interest do not contain a polyA tract, one can be attached by polymerization with terminal transferase, or via ligation of an oligoA linker, as is known in the art.

Alternatively, chemical crosslinking may be done, for example by photoactivated crosslinking of thymidine to reactive groups, as is known in the art.

In general, special methods are required to decode clonal arrays, as is more fully outlined below.

As described above generally for proteins, nucleic acid bioactive agents may be naturally occurring nucleic acids, random nucleic acids, or "biased" random nucleic acids. For example, digests of procaryotic or eukaryotic genomes may be used as is outlined above for proteins.

In general, probes of the present invention are designed to be complementary to a target sequence (either the target analyte sequence of the sample or to other probe sequences, as is described herein), such that hybridization of the target and the probes of the present invention occurs. This complementarily need not be perfect; there may be any number of base pair mismatches that will interfere with hybridization between the target sequence and the single stranded nucleic acids of the present invention. However, if the number of mutations is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. Thus, by "substantially complementary" herein is meant that the probes are sufficiently complementary to the target sequences to hybridize under the selected reaction conditions. High stringency conditions are known in the art; see for example Maniatis et al., *Molecular Cloning: A Laboratory Manual*, 2d Edition, 1989, and *Short Protocols in Molecular Biology*, ed. Ausubel, et al., both of which are hereby incorporated by reference. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10 C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are

occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30 C for short probes (e.g. 10 to 50 nucleotides) and at least about 60 C for long probes (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. In another embodiment, less stringent hybridization conditions are used; for example, moderate or low stringency conditions may be used, as are known in the art; see Maniatis and Ausubel, supra, and Tijssen, supra.

The term 'target sequence" or grammatical equivalents herein means a nucleic acid sequence on a single strand of nucleic acid. The target sequence may be a portion of a gene, a regulatory sequence, genomic DNA, cDNA, RNA including mRNA and rRNA, or others. It may be any length, with the understanding that longer sequences are more specific. As will be appreciated by those in the art, the complementary target sequence may take many forms. For example, it may be contained within a larger nucleic acid sequence, i.e. all or part of a gene or mRNA, a restriction fragment of a plasmid or genomic DNA, among others. As is outlined more fully below, probes are made to hybridize to target sequences to determine the presence or absence of the target sequence in a sample. Generally speaking, this term will be understood by those skilled in the art.

In a preferred embodiment, the bioactive agents are organic chemical moieties, a wide variety of which are available in the literature.

In a preferred embodiment, each bead comprises a single type of bioactive agent, although a plurality of individual bioactive agents are preferably attached to each bead. Similarly, preferred embodiments utilize more than one microsphere containing a unique bioactive agent; that is, there is redundancy built into the system by the use of subpopulations of microspheres, each microsphere in the subpopulation containing the same bioactive agent.

As will be appreciated by those in the art, the bioactive agents may either be synthesized directly on the beads, or they may be made and then attached after synthesis. In a preferred embodiment, linkers are used to attach the bioactive agents to the beads, to allow both good attachment, sufficient flexibility to allow good interaction with the target molecule, and to avoid undesirable binding reactions.

In a preferred embodiment, the bioactive agents are synthesized directly on the beads. As is known in the art, many classes of chemical compounds are currently synthesized on solid supports, including beads, such as peptides, organic moieties, and nucleic acids.

In a preferred embodiment, the bioactive agents are synthesized first, and then covalently attached to the beads. As will be appreciated by those in the art, this will be done depending on the composition

of the bioactive agents and the beads. The functionalization of solid support surfaces such as certain polymers with chemically reactive groups such as thiols, amines, carboxyls, etc. is generally known in the art. Accordingly, "blank" microspheres may be used that have surface chemistries that facilitate the attachment of the desired functionality by the user. Some examples of these surface chemistries for blank microspheres include, but are not limited to, amino groups including aliphatic and aromatic amines, carboxylic acids, aldehydes, amides, chloromethyl groups, hydrazide, hydroxyl groups, sulfonates and sulfates.

These functional groups can be used to add any number of different candidate agents to the beads, generally using known chemistries. For example, candidate agents containing carbohydrates may be attached to an amino-functionalized support; the aldehyde of the carbohydrate is made using standard techniques, and then the aldehyde is reacted with an amino group on the surface. In an alternative embodiment, a sulfhydryl linker may be used. There are a number of sulfhydryl reactive linkers known in the art such as SPDP, maleimides, -haloacetyls, and pyridyl disulfides (see for example the 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference) which can be used to attach cysteine containing proteinaceous agents to the support. Alternatively, an amino group on the candidate agent may be used for attachment to an amino group on the surface. For example, a large number of stable bifunctional groups are well known in the art, including homobifunctional and heterobifunctional linkers (see Pierce Catalog and Handbook, pages 155-200). In an additional embodiment, carboxyl groups (either from the surface or from the candidate agent) may be derivatized using well known linkers (see the Pierce catalog). For example, carbodiimides activate carboxyl groups for attack by good nucleophiles such as amines (see Torchilin et al., Critical Rev. Therapeutic Drug Carrier Systems, 7(4):275-308 (1991), expressly incorporated herein). Proteinaceous candidate agents may also be attached using other techniques known in the art, for example for the attachment of antibodies to polymers; see Slinkin et al., Bioconj. Chem. 2:342-348 (1991); Torchilin et al., *supra*; Trubetskoy et al., Bioconj. Chem. 3:323-327 (1992); King et al., Cancer Res. 54:6176-6185 (1994); and Wilbur et al., Bioconjugate Chem. 5:220-235 (1994), all of which are hereby expressly incorporated by reference). It should be understood that the candidate agents may be attached in a variety of ways, including those listed above. Preferably, the manner of attachment does not significantly alter the functionality of the candidate agent; that is, the candidate agent should be attached in such a flexible manner as to allow its interaction with a target.

Specific techniques for immobilizing enzymes on microspheres are known in the prior art. In one case, NH_2 surface chemistry microspheres are used. Surface activation is achieved with a 2.5% glutaraldehyde in phosphate buffered saline (10 mM) providing a pH of 6.9. (138 mM NaCl, 2.7 mM, KCl). This is stirred on a stir bed for approximately 2 hours at room temperature. The microspheres are then rinsed with ultrapure water plus 0.01% tween 20 (surfactant) -0.02%, and rinsed again with a

pH 7.7 PBS plus 0.01% tween 20. Finally, the enzyme is added to the solution, preferably after being prefiltered using a 0.45 m amicon micropure filter.

In some embodiments, the microspheres may additionally comprise identifier binding ligands for use in certain decoding systems. By "identifier binding ligands" or "IBLs" herein is meant a compound that will specifically bind a corresponding decoder binding ligand (DBL) to facilitate the elucidation of the identity of the bioactive agent attached to the bead. That is, the IBL and the corresponding DBL form a binding partner pair. By "specifically bind" herein is meant that the IBL binds its DBL with specificity sufficient to differentiate between the corresponding DBL and other DBLs (that is, DBLs for other IBLs), or other components or contaminants of the system. The binding should be sufficient to remain bound under the conditions of the decoding step, including wash steps to remove non-specific binding. In some embodiments, for example when the IBLs and corresponding DBLs are proteins or nucleic acids, the dissociation constants of the IBL to its DBL will be less than about 10^{-4}-10^{-6} M^{-1}, with less than about 10^{-5} to 10^{-9} M^{-1} being preferred and less than about 10^{-7} -10^{-9} M^{-1} being particularly preferred.

IBL-DBL binding pairs are known or can be readily found using known techniques. For example, when the IBL is a protein, the DBLs include proteins (particularly including antibodies or fragments thereof (FAbs, etc.)) or small molecules, or vice versa (the IBL is an antibody and the DBL is a protein). Metal ion- metal ion ligands or chelators pairs are also useful. Antigen-antibody pairs, enzymes and substrates or inhibitors, other protein-protein interacting pairs, receptor-ligands, complementary nucleic acids (including nucleic acid molecules that form triple helices), and carbohydrates and their binding partners are also suitable binding pairs. Nucleic acid - nucleic acid binding proteins pairs are also useful, including single-stranded or double-stranded nucleic acid binding proteins, and small molecule nucleic acid binding agents. Similarly, as is generally described in U.S. Patents 5,270,163, 5,475,096, 5,567,588, 5,595,877, 5,637,459, 5,683,867,5,705,337, and related patents, hereby incorporated by reference, nucleic acid "aptamers" can be developed for binding to virtually any target; such an aptamer-target pair can be used as the IBL-DBL pair. Similarly, there is a wide body of literature relating to the development of binding pairs based on combinatorial chemistry methods.

In a preferred embodiment, the IBL is a protein, particularly an enzyme, that is conjugated to a bead. One advantage of using proteins/enzymes as IBLs is that many proteins and enzymes are commercially available in relatively pure preparations. Proteins contain many suitable functional groups for attachment to beads. Moreover, the identity of many small molecules including substrates that bind the proteins/enzymes are known. Such small molecules serve as the DBL. In addition, the small molecule can serve as the IBL and the enzyme as the DBL. As will be appreciated by those in the art, combinations of enzymes and small molecules can be used as well, or multiple enzymes and small molecules. Another potential advantage relates to the purification of the labeled proteins. If one

conjugates a fluorophore to a recrystallizable protein, then the unlabeled protein can be removed from labeled protein by use of crystallization. By filtering off the crystalline solid, the labeled protein will be concentrated in the filtrate.

In an alternative embodiment, antigen-antibody pairs are used as DBL-IBL combinations. In one embodiment antibodies are conjugated to different labels, defined below, that are then used to decode antigen-labeled beads.

In a preferred embodiment, the IBL is a molecule whose color or luminescence properties change in the presence of a selectively-binding DBL.

In one embodiment, the DBL may be attached to a bead, i.e. a "decoder bead", that may carry a label such as a fluorophore.

In a preferred embodiment, the IBL-DBL pair comprise substantially complementary single-stranded nucleic acids. In this embodiment, the binding ligands can be referred to as "identifier probes" and "decoder probes". Generally, the identifier and decoder probes range from about 4 basepairs in length to about 1000, with from about 6 to about 100 being preferred, and from about 8 to about 40 being particularly preferred. What is important is that the probes are long enough to be specific, i.e. to distinguish between different IBL-DBL pairs, yet short enough to allow both a) dissociation, if necessary, under suitable experimental conditions, and b) efficient hybridization.

In a preferred embodiment, as is more fully outlined below, the IBLs do not bind to DBLs. Rather, the IBLs are used as identifier moieties ("IMs") that are identified directly, for example through the use of mass spectroscopy.

Alternatively, in a preferred embodiment, the IBL and the bioactive agent are the same moiety; thus, for example, as outlined herein, particularly when no optical signatures are used, the bioactive agent can serve as both the identifier and the agent. For example, in the case of nucleic acids, the bead-bound probe (which serves as the bioactive agent) can also bind decoder probes, to identify the sequence of the probe on the bead. Thus, in this embodiment, the DBLs bind to the bioactive agents. This is particularly useful as this embodiment can give information about the array or the assay in addition to decoding. For example, as is more fully described below, the use of the DBLs allows array calibration and assay development. This may be done even if the DBLs are not used as such; for example in non-random arrays, the use of these probe sets can allow array calibration and assay development even if decoding is not required.

In a preferred embodiment, the microspheres do not contain an optical signature. That is, as outlined in U.S.S.N.s 08/818,199 and 09/151,877, previous work had each subpopulation of microspheres comprising a unique optical signature or optical tag that is used to identify the unique bioactive agent of that subpopulation of microspheres; that is, decoding utilizes optical properties of the beads such that a bead comprising the unique optical signature may be distinguished from beads at other locations with different optical signatures. Thus the previous work assigned each bioactive agent a unique optical signature such that any microspheres comprising that bioactive agent are identifiable on the basis of the signature. These optical signatures comprised dyes, usually chromophores or fluorophores, that were entrapped or attached to the beads themselves. Diversity of optical signatures utilized different fluorochromes, different ratios of mixtures of fluorochromes, and different concentrations (intensities) of fluorochromes.

Thus, the present invention does not rely solely on the use of optical properties to decode the arrays. However, as will be appreciated by those in the art, it is possible in some embodiments to utilize optical signatures as an additional coding method, in conjunction with the present system. Thus, for example, as is more fully outlined below, the size of the array may be effectively increased while using a single set of decoding moieties in several ways, one of which is the use in combination with optical signatures one beads. Thus, for example, using one "set" of decoding molecules, the use of two populations of beads, one with an optical signature and one without, allows the effective doubling of the array size. The use of multiple optical signatures similarly increases the possible size of the array.

In a preferred embodiment, each subpopulation of beads comprises a plurality of different IBLs. By using a plurality of different IBLs to encode each bioactive agent, the number of possible unique codes is substantially increased. That is, by using one unique IBL per bioactive agent, the size of the array will be the number of unique IBLs (assuming no "reuse" occurs, as outlined below). However, by using a plurality of different IBLs per bead, n, the size of the array can be increased to 2^n, when the presence or absence of each IBL is used as the indicator. For example, the assignment of 10 IBLs per bead generates a 10 bit binary code, where each bit can be designated as "1" (IBL is present) or "0" (IBL is absent). A 10 bit binary code has 2^{10} possible variants However, as is more fully discussed below, the size of the array may be further increased if another parameter is included such as concentration or intensity; thus for example, if two different concentrations of the IBL are used, then the array size increases as 3^n. Thus, in this embodiment, each individual bioactive agent in the array is assigned a combination of IBLs, which can be added to the beads prior to the addition of the bioactive agent, after, or during the synthesis of the bioactive agent, i.e. simultaneous addition of IBLs and bioactive agent components.

Alternatively, when the bioactive agent is a polymer of different residues, i.e. when the bioactive agent is a protein or nucleic acid, the combination of different IBLs can be used to elucidate the sequence of the protein or nucleic acid.

Thus, for example, using two different IBLs (IBL1 and IBL2), the first position of a nucleic acid can be elucidated: for example, adenosine can be represented by the presence of both IBL1 and IBL2; thymidine can be represented by the presence of IBL1 but not IBL2, cytosine can be represented by the presence of IBL2 but not IBL1, and guanosine can be represented by the absence of both. The second position of the nucleic acid can be done in a similar manner using IBL3 and IBL4; thus, the presence of IBL1, IBL2, IBL3 and IBL4 gives a sequence of AA; IBL1, IBL2, and IBL3 shows the sequence AT; IBL1, IBL3 and IBL4 gives the sequence TA, etc. The third position utilizes IBL5 and IBL6, etc. In this way, the use of 20 different identifiers can yield a unique code for every possible 10-mer.

The system is similar for proteins but requires a larger number of different IBLs to identify each position, depending on the allowed diversity at each position. Thus for example, if every amino acid is allowed at every position, five different IBLs are required for each position. However, as outlined above, for example when using random peptides as the bioactive agents, there may be bias built into the system; not all amino acids may be present at all positions, and some positions may be preset; accordingly, it may be possible to utilize four different IBLs for each amino acid.

In this way, a sort of "bar code" for each sequence can be constructed; the presence or absence of each distinct IBL will allow the identification of each bioactive agent.

In addition, the use of different concentrations or densities of IBLs allows a "reuse" of sorts. If, for example, the bead comprising a first agent has a 1X concentration of IBL, and a second bead comprising a second agent has a 10X concentration of IBL, using saturating concentrations of the corresponding labelled DBL allows the user to distinguish between the two beads.

In addition, the use of different ratios of labeled to unlabeled IBLs allows for a differentiation of the intensity rather than the concentration of IBLs. For example, if a bead comprising a first agent has a ratio of 1:1 (labeled: unlabeled IBL), while a second bead comprising a second agent has a ration of 1:10 (labeled: unlabeled IBL), the user will distinguish the different intensities between the two beads. This procedure offers the advantage that a broad spectrum of intensities of labels can be created using only a single label.

Once the microspheres comprising the candidate agents and the unique IBLs are generated, they are added to the substrate to form an array. It should be noted that while most of the methods described

herein add the beads to the substrate prior to the assay, the order of making, using and decoding the array can vary. For example, the array can be made, decoded, and then the assay done. Alternatively, the array can be made, used in an assay, and then decoded; this may find particular use when only a few beads need be decoded. Alternatively, the beads can be added to the assay mixture, i.e. the sample containing the target analytes, prior to the addition of the beads to the substrate; after addition and assay, the array may be decoded. This is particularly preferred when the sample comprising the beads is agitated or mixed; this can increase the amount of target analyte bound to the beads per unit time, and thus (in the case of nucleic acid assays) increase the hybridization kinetics. This may find particular use in cases where the concentration of target analyte in the sample is low; generally, for low concentrations, long binding times must be used.

In addition, adding the beads to the assay mixture can allow sorting or selection. For example, a large library of beads may be added to a sample, and only those beads that bind the sample may be added to the substrate. For example, if the target analyte is fluorescently labeled (either directly (for example by the incorporation of labels into nucleic acid amplification reactions) or indirectly (for example via the use of sandwich assays)), beads that exhibit fluorescence as a result of target analyte binding can be sorted via Fluorescence Activated Cell Sorting (FACS) and only these beads added to an array and subsequently decoded. Similarly, the sorting may be accomplished through affinity techniques; affinity columns comprising the target analytes can be made, and only those beads which bind are used on the array. Similarly, two bead systems can be used; for example, magnetic beads comprising the target analytes can be used to "pull out" those beads that will bind to the targets, followed by subsequent release of the magnetic beads (for example via temperature elevation) and addition to an array.

In general, the methods of making the arrays and of decoding the arrays is done to maximize the number of different candidate agents that can be uniquely encoded. The compositions of the invention may be made in a variety of ways. In general, the arrays are made by adding a solution or slurry comprising the beads to a surface containing the sites for association of the beads. This may be done in a variety of buffers, including aqueous and organic solvents, and mixtures. The solvent can evaporate, and excess beads removed.

In a preferred embodiment, when non-covalent methods are used to associate the beads to the array, a novel method of loading the beads onto the array is used. This method comprises exposing the array to a solution of particles (including microspheres and cells) and then applying energy, e.g. agitating or vibrating the mixture. This results in an array comprising more tightly associated particles, as the agitation is done with sufficient energy to cause weakly-associated beads to fall off (or out, in the case of wells). These sites are then available to bind a different bead. In this way, beads that exhibit a high affinity for the sites are selected. Arrays made in this way have two main advantages as

compared to a more static loading: first of all, a higher percentage of the sites can be filled easily, and secondly, the arrays thus loaded show a substantial decrease in bead loss during assays. Thus, in a preferred embodiment, these methods are used to generate arrays that have at least about 50% of the sites filled, with at least about 75% being preferred, and at least about 90% being particularly preferred. Similarly, arrays generated in this manner preferably lose less than about 20% of the beads during an assay, with less than about 10% being preferred and less than about 5% being particularly preferred.

In this embodiment, the substrate comprising the surface with the discrete sites is immersed into a solution comprising the particles (beads, cells, etc.). The surface may comprise wells, as is described herein, or other types of sites on a patterned surface such that there is a differential affinity for the sites. This differnetial affinity results in a competitive process, such that particles that will associate more tightly are selected. Preferably, the entire surface to be "loaded" with beads is in fluid contact with the solution. This solution is generally a slurry ranging from about 10,000:1 beads:solution (vol:vol) to 1:1. Generally, the solution can comprise any number of reagents, including aqueous buffers, organic solvents, salts, other reagent components, etc. In addition, the solution preferably comprises an excess of beads; that is, there are more beads than sites on the array. Preferred embodiments utilize two-fold to billion-fold excess of beads.

The immersion can mimic the assay conditions; for example, if the array is to be "dipped" from above into a microtiter plate comprising samples, this configuration can be repeated for the loading, thus minimizing the beads that are likely to fall out due to gravity.

Once the surface has been immersed, the substrate, the solution, or both are subjected to a competitive process, whereby the particles with lower affinity can be disassociated from the substrate and replaced by particles exhibiting a higher affinity to the site. This competitive process is done by the introduction of energy, in the form of heat, sonication, stirring or mixing, vibrating or agitating the solution or substrate, or both.

A preferred embodiment utilizes agitation or vibration. In general, the amount of manipulation of the substrate is minimized to prevent damage to the array; thus, preferred embodiments utilize the agitation of the solution rather than the array, although either will work. As will be appreciated by those in the art, this agitation can take on any number of forms, with a preferred embodiment utilizing microtiter plates comprising bead solutions being agitated using microtiter plate shakers.

The agitation proceeds for a period of time sufficient to load the array to a desired fill. Depending on the size and concentration of the beads and the size of the array, this time may range from about 1 second to days, with from about 1 minute to about 24 hours being preferred.

In a preferred embodiment, the substrate is pressed into a mixture of dry beads (although slurries may be used as well), and then tapped to remove excess beads.

It should be noted that not all sites of an array may comprise a bead; that is, there may be some sites on the substrate surface which are empty. In addition, there may be some sites that contain more than one bead, although this is not preferred.

In some embodiments, for example when chemical attachment is done, it is possible to associate the beads in a non-random or ordered way. For example, using photoactivatible attachment linkers or photoactivatible adhesives or masks, selected sites on the array may be sequentially rendered suitable for attachment, such that defined populations of beads are laid down.

The arrays of the present invention are constructed such that information about the identity of the candidate agent is built into the array, such that the random deposition of the beads in the fiber wells can be "decoded" to allow identification of the candidate agent at all positions. This may be done in a variety of ways, and either before, during or after the use of the array to detect target molecules.

Thus, after the array is made, it is "decoded" in order to identify the location of one or more of the bioactive agents, i.e. each subpopulation of beads, on the substrate surface.

In a preferred embodiment, a selective decoding system is used. In this case, only those microspheres exhibiting a change in the optical signal as a result of the binding of a target analyte are decoded. This is commonly done when the number of "hits", i.e. the number of sites to decode, is generally low. That is, the array is first scanned under experimental conditions in the absence of the target analytes. The sample containing the target analytes is added, and only those locations exhibiting a change in the optical signal are decoded. For example, the beads at either the positive or negative signal locations may be either selectively tagged or released from the array (for example through the use of photocleavable linkers), and subsequently sorted or enriched in a fluorescence-activated cell sorter (FACS). That is, either all the negative beads are released, and then the positive beads are either released or analyzed in situ, or alternatively all the positives are released and analyzed. Alternatively, the labels may comprise halogenated aromatic compounds, and detection of the label is done using for example gas chromatography, chemical tags, isotopic tags, or mass spectral tags.

As will be appreciated by those in the art, this may also be done in systems where the array is not decoded; i.e. there need not ever be a correlation of bead composition with location. In this embodiment, the beads are loaded on the array, and the assay is run. The "positives", i.e. those beads displaying a change in the optical signal as is more fully outlined below, are then "marked" to

distinguish or separate them from the "negative" beads. This can be done in several ways, preferably using fiber optic arrays. In a preferred embodiment, each bead contains a fluorescent dye. After the assay and the identification of the "positives" or "active beads", light is shown down either only the positive fibers or only the negative fibers, generally in the presence of a light-activated reagent (typically dissolved oxygen). In the former case, all the active beads are photobleached. Thus, upon non-selective release of all the beads with subsequent sorting, for example using a fluorescence activated cell sorter (FACS) machine, the non-fluorescent active beads can be sorted from the fluorescent negative beads. Alternatively, when light is shown down the negative fibers, all the negatives are non-fluorescent and the the postives are fluorescent, and sorting can proceed. The characterization of the attached bioactive agent may be done directly, for example using mass spectroscopy.

Alternatively, the identification may occur through the use of identifier moieties ("IMs"), which are similar to IBLs but need not necessarily bind to DBLs. That is, rather than elucidate the structure of the bioactive agent directly, the composition of the IMs may serve as the identifier. Thus, for example, a specific combination of IMs can serve to code the bead, and be used to identify the agent on the bead upon release from the bead followed by subsequent analysis, for example using a gas chromatograph or mass spectroscope.

Alternatively, rather than having each bead contain a fluorescent dye, each bead comprises a non-fluorescent precursor to a fluorescent dye. For example, using photocleavable protecting groups, such as certain ortho-nitrobenzyl groups, on a fluorescent molecule, photoactivation of the fluorochrome can be done. After the assay, light is shown down again either the "positive" or the "negative" fibers, to distinguish these populations. The illuminated precursors are then chemically converted to a fluorescent dye. All the beads are then released from the array, with sorting, to form populations of fluorescent and non-fluorescent beads (either the positives and the negatives or vice versa).

In an alternate preferred embodiment, the sites of association of the beads (for example the wells) include a photopolymerizable reagent, or the photopolymerizable agent is added to the assembled array. After the test assay is run, light is shown down again either the "positive" or the "negative" fibers, to distinquish these populations. As a result of the irradiation, either all the positives or all the negatives are polymerized and trapped or bound to the sites, while the other population of beads can be released from the array.

In a preferred embodiment, the location of every bioactive agent is determined using decoder binding ligands (DBLs). As outlined above, DBLs are binding ligands that will either bind to identifier binding ligands, if present, or to the bioactive agents themselves, preferably when the bioactive agent is a nucleic acid or protein.

In a preferred embodiment, as outlined above, the DBL binds to the IBL.

In a preferred embodiment, the bioactive agents are single-stranded nucleic acids and the DBL is a substantially complementary single-stranded nucleic acid that binds (hybridizes) to the bioactive agent, termed a decoder probe herein. A decoder probe that is substantially complementary to each candidate probe is made and used to decode the array. In this embodiment, the candidate probes and the decoder probes should be of sufficient length (and the decoding step run under suitable conditions) to allow specificity; i.e. each candidate probe binds to its corresponding decoder probe with sufficient specificity to allow the distinction of each candidate probe.

In a preferred embodiment, the DBLs are either directly or indirectly labeled. By "labeled" herein is meant that a compound has at least one element, isotope or chemical compound attached to enable the detection of the compound. In general, labels fall into three classes: a) isotopic labels, which may be radioactive or heavy isotopes; b) magnetic, electrical, thermal; and c) colored or luminescent dyes; although labels include enzymes and particles such as magnetic particles as well. Examples of such lables include: peroxidase; alkaline phosphatase; biotin; urease; E alactosidase; BSA/KLH; gold particles; quantum dots; redox indicators; pH indicators; E-lactamase; luciferase; TSA; SPA; chemiluminescence; sonoluminescence; fluorophores; phosphors; and, fluorescent metal ion sensors. Preferred labels include luminescent labels. In a preferred embodiment, the DBL is directly labeled, that is, the DBL comprises a label. In an alternate embodiment, the DBL is indirectly labeled; that is, a labeling binding ligand (LBL) that will bind to the DBL is used. In this embodiment, the labeling binding ligand-DBL pair can be as described above for IBL-DBL pairs. Suitable labels include, but are not limited to, fluorescent lanthanide complexes, including those of Europium and Terbium, fluorescein, rhodamine, tetramethylrhodamine, eosin, erythrosin, coumarin, methyl-coumarins, pyrene, Malacite green, stilbene, Lucifer Yellow, Cascade Blue™, Texas Red, FITC, PE, cy3, cy5 and others described in the 6th Edition of the Molecular Probes Handbook by Richard P. Haugland, hereby expressly incorporated by reference. In addition, ion-sensitive dyes such as bis-BITC, Newport green, fluo 3, fura red find use in the invention.

In one embodiment, the label is a molecule whose color or luminescence properties change in the presence of the IBL, due to a change in the local environment. For example, the label may be: (1) a fluorescent pH indicator whose emission intensity changes with pH; (2) a fluorescent ion indicator, whose emission properties change with ion concentration; or (3) a fluorescent molecule such as an ethidium salt whose fluorescence intensity increases in hydrophobic environments.

In one embodiment ion sensitive dyes are used for decoding. That is, dyes that fluoresce in the presence of certain ions are used. In a preferred embodiment the ion sensitive dyes are attached to the beads. An advantage of attaching dyes directly to microspheres is that the cost associated with oligonucleotide synthesis is avoided.

Accordingly, when such dyes are attached to the beads they can be used to encode a specific nucleotide sequence, or the identity of the bioactive agent. Once the beads are distributed on the array, the array is contacted with buffers that include certain ions. In the presence of appropriate ion, certain dyes will fluoresce and fluorescence is detected. In a preferred embodiment, the array is contacted with different buffers sequentially. Each buffer contains a different ion. Preferably the array is washed between buffer solutions so that any signal produced as a result of the contact with the ion solution is diminished.

In this embodiment multiple different ion sensitive dyes can be used. That is, preferably, more than 2 with more than 3 or 4 dyes are attached to the microspheres. In some embodiments, more than 10 dyes are attached to the microsphere.

One advantage to using ion sensitive dyes is that fluorescence bleed-through is diminished. That is, because the response of the dyes is either positive or negative, i.e. they interact or they don't, bleed-through is avoided.

In a preferred embodiment, decoding of self-assembled random arrays is done on the basis of pH titration. In this embodiment, in addition to bioactive agents, the beads comprise optical signatures, wherein the optical signatures are generated by the use of pH-responsive dyes (sometimes referred to herein as "pH dyes") such as fluorophores. This embodiment is similar to that outlined in PCT US98/05025 and U.S.S.N. 09/151,877, both of which are expressly incorporated by reference, except that the dyes used in the present invention exhibits changes in fluorescence intensity (or other properties) when the solution pH is adjusted from below the pKa to above the pKa (or vice versa). In a preferred embodiment, a set of pH dyes is used, each with a different pKa, preferably separated by at least 0.5 pH units. Preferred embodiments utilize a pH dye set of pKa's of 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11, and 11.5. Each bead can contain any subset of the pH dyes, and in this way a unique code for the bioactive agent is generated. Thus, the decoding of an array is achieved by titrating the array from pH 1 to pH 13, and measuring the fluorescence signal from each bead as a function of solution pH.

In one embodiment, the decoder probes are labeled in situ; that is, they need not be labeled prior to the decoding reaction. In this embodiment, the incoming decoder probe is shorter than the candidate probe, creating a 5' "overhang" on the decoding probe. The addition of labeled ddNTPs (each labeled with a unique tag) and a polymerase will allow the addition of the tags in a sequence specific manner, thus creating a sequence-specific pattern of signals. Similarly, other modifications can be done, including ligation, etc.

In one embodiment, a series of sequential extension and denaturing stages allows for positional decoding of the beads. That is, following one round of extension, the DBL is removed or stripped from

the IBL and a second round of extension is performed. The contents of any given primer extension reaction is shown in Figure 8.

In the primer extension method, up to four fluorescently-labeled dideoxynucleotide terminators each with a different colored label (e.g. using ABI's dRhodamine® terminators such as dR110,dR6G, dTAMRA, dROX or Big Dye® Terminators) could be used for 'painting'. The number of unique beads that can be decoded is given by $N= y^x$, where n= the number of unique codes, y= the number of colors and x= the number of stages or primer extension reactions. This formula yields 4096 unique codes using 6 primer extension stages and four colors (i.e. $4096 = 4^6$).

In a preferred embodiment the bioactive agent is synthesized with encoding sequences that allows decoding to be accomplished independent of the useable portion of the bioactive agent, i.e. nucleic acid sequence. By "useable" portion is meant the adapter sequence or target sequence. In a preferred embodiment the bioactive agent also is synthesized with a primer region to facilitate sequencing or primer extension analysis of the encoding sequence (see Figure 12). As shown in Figure 12, a primer complementary to a portion of the ssDNA on the bead is extended by single base polymerase extension using dye-labeled chain terminating nucleotides. After incorporation of a labeled, i.e. fluorescent, chain terminator and reading of the array, i.e. detecting the label, the fluorescence is removed. Removal of the fluorescence is accomplished directly when reversible chain terminators are used (see Canard and Sarfati, Gene, 148(1): 1-6, 1994, which is expressly incorporated herein by reference). However, in a preferred embodiment the primer is dissociated from the bead and a subsequent primer is annealed. Preferably the subsequent primer is 1 nucleotide longer than the former primer so that an additional nucleotide in the encoding sequence can be interrogated. In a preferred embodiment the subsequent primer is lengthened by the addition of a degenerate base. This can be accomplished either by incorporating all 4 nucleotides into the additional position of the primer, or by the incorporation of a universal base into the primer. The new primer is then subject to extension as described above. The primer extension can be repeated as many times as are necessary to decode the microsphere.

Decoding by sequencing has the advantage that encoding a vast number of distinct codes by using only a small number of nucleotides. That is, the information content of the encoding region depends on the number of combinations of the bases used, i.e. 4 when A, C, G and T are used, that are present at each position and the length of the encoding region. When 4 nucleotides are used, there are up to fifteen possible combinations of nucleotides at each position (A, C, G, T, A/C, A/G, A/T, C/G, C/T, G/T, A/C/G, A/C/T, A/G/T, C/G/G and A/C/G/T). As such, the information content is 15^n, where n is equal to the length of the encoding sequence. As such, for example, with just 3 base positions, 3375 different codes can be generated.

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In addition an additional preferred embodiment includes the combination of decoding by sequencing with decoding by hybridization. In one application of decoding by sequencing, universal primers, each one base longer than the previous, are sequentially hybridized to the array. The base adjacent to the 3' end of the universal primer is determined by a single base extension reaction employing labeled-chain terminators, preferably using four different color labels one for each base. Since a hybridization event must be performed for each base read, once can improve the decoding power by using the hybridization event to also decode. However, rather than using universal primers, zipcode sequences would be used instead (see Figure 13)

The placement of the encoding sequences can be either on the "bead-side" of the gene-specific sequence or on the terminal side. Placement of the encoding sequences on the terminal side offers that advantage of incorporating a cleavable linker between the encoding sequences and the gene-specific sequence (Fig. 14). After completion of decoding, the encoding sequences can be removed to prevent possible interference in the analytical assay.

In one embodiment following decoding, the label is extinguished. By "extinguished" is meant that the signal from the label is eliminated. This is accomplished by a variety of techniques including cleaving the label or destroying the signal of the label. That is, in one embodiment, the label is attached to the DBL via a cleavable linker. Following a decoding step, the linker is cleaved thereby releasing the label. In one embodiment cleavage of the label results in deprotection of the bioactive agent, for example when the bioactive agent contains a protecting group. Alternatively, cleavage of the label preserves the nature of the bioactive agent. That is, what is important is that cleavage of the label does not interfere with the bioactive agent.

In an alternative embodiment, the label is not cleaved, but rather is eliminated by destroying the signal of the label. That is, following decoding, the label is contacted with an agent that degrades or destroys the signal. For example, organic fluorescent dyes are subject to chemical degradation under appropriate conditions. Thus, following a decoding step where the fluorescent signal of the dyes are detected, the fluorophore is destroyed by an appropriate chemical solution that degrades the fluorescent molecule sufficient leaving no trace of fluorescence. In this way, beads are tagged with fluorescent labels, i.e. barcodes of sorts, their location or identity deciphered and recorded. The beads are then exposed to the "fluorescent destroying solution" and the fluorescence is eliminated, allowing an analytical assay to be conducted. Suitable fluorescent dyes include but are not limited to various bodipy, or Alexa dyes from Molecular Probes. The "fluorescent destroying solution" is a reducing or oxidizing solution, such as 0.01 SM sodium periodate.

In a preferred embodiment not only is the label cleaved, but the encoding nucleic acid is cleaved. This method finds particular use in removing residual signal following a decoding step. Accordingly, in a preferred embodiment a cleavable linker can be used to bridge the adapter and beads. After

decoding, the adapter oligonucleotide(s) can be easily removed through cleavable linkers. There are many commercially available cleavable linkers. They are routinely used in the combinatorial solid-phase synthesis field. The linkers can be cleaved under acid, base or photo radiation conditions.

In addition, restriction enzymes can be used to cleave a linker. Alternatively, because of the sequence specificity of restriction enzymes, they also are used to cleave encoding nucleic acids. A change in signal following incubation with a sequence specific enzyme decodes the bead. An example is illustrative.

Consider the decoding of 6 bead types with three zip codes or adapters. In general, the strategy will have B=(2^n)-2. Where B is the number of types encoded and n is the number of zip codes. The bead types are z1, z2, z3, z1z2, z1z3, and z2z3. Here zizj indicates that both codes are on a bead. Every code is already dye labeled.

Generally,each code is designed with a site that can be specifically cut with some agent. This includes, but is not limited to, restriction enzymes. For ease of description, the process using restriction enzymes and zip codes that contain specific restriction sites is described. When restriction enzymes are used, the zip codes are double stranded. Each stage would consist of sequentially adding one restriction enzyme. An image is taken prior to stage 1, and the intensity of every bead is measured. Restriction enzyme 1 (Re1) is added. New intensity measurements are taken. The information of which beads decrease in intensity, and which remain the same. Detecting the pattern of beads for which the signal "decreased" and "stayed the same" uniquely determines the bead type. If we denote "decreased" by 1 and "stayed the same" by 0, then the decoding would give the following codes:

z1 : 1,0,0
z2 : 0,1,0
z3 : 0,0,1
z1z2 : 1,1,0
z1z3 : 1,0,1
z2z3 : 0,1,1.

The general approach uses n codes, which would give (2^n)-2 types. Subtracting 2 leaves off the all off state, and the all zip codes used state.

In another embodiment the array is decoded by using exonuclease enzyme. In this embodiment the microspheres are encoded with dye-labeled nucleotides. Preferably each nucleotide is labeled with a discrete label. In this particular embodiment it is desirable to protect the bioactive agent from cleavage. This can be accomplished by a variety of known methods including hybridizing the bioactive agent to a complementary sequence or blocking the terminus of the bioactive agent. Alternatively, if

the exonuclease enzyme operates from 5' to 3', one can design zipcodes from 5' to 3' and SNP-gene specific oligos from 3' to 5'. This way zipcode sequences will be operated on by the exonuclease, but the SNP/gene-specific sequences will remain intact.

The microsphere is then contacted with an exonuclease that successively removes the labeled nucleotides of the encoding sequence. Monitoring of the signal of the microspheres should begin upon addition of the exonuclease and monitored in all channels that correspond to the signal emitted by the labels in use. For example, assume the microsphere has all the zipcodes available (with the above specifications), and is in a solution. At time T=0, the exonuclease enzyme is applied to the solution. At the same time, the acquisition system (CCD) starts taking images every few seconds (Figure 15). The assumption is that the reaction of the exonuclease is slow enough, so one would have ample time to take several images, as the reaction is proceeding. If this is not the case, the reaction can be slowed down by increasing the stringency conditions.

The exonuclease removes the nucleotides, one at a time. Therefore, ideally, one would notice a step reduction in the signal of the appropriate channel, once the nucleotide is removed (and moved away from the focal zone). The complete set of waveforms (i.e., the four color channels, and from T0 to Tend) will provide a unique signature for every zipcode.

In practice, the removal of the nucleotides will not be completely synchronized on different oligos (as shown in Figure 15). For instance, for two oligos on the same bead (same zipcode), the exonuclease may have removed one nucleotide from one, and two nucleotides from the other. However, since one monitors many oligos from each bead, the overall trend should be preserved, i.e., in general the nucleotides closer to the end should be removed first. This means that in general, although the transitions on the waveforms may not be sharp, the signatures could still be unique.

Accordingly, the identification of the location of the individual beads (or subpopulations of beads) is done using one or more decoding steps comprising a binding between the labeled DBL and either the IBL or the bioactive agent (i.e. a hybridization between the candidate probe (sometimes referred to herein as a "decoding sequence) and the decoder probe when the bioactive agent is a nucleic acid). After decoding, the DBLs can be removed and the array can be used; however, in some circumstances, for example when the DBL binds to an IBL and not to the bioactive agent, the removal of the DBL is not required (although it may be desirable in some circumstances). In addition, as outlined herein, decoding may be done either before the array is used in an assay, during the assay, or after the assay.

In one embodiment, a single decoding step is done. In this embodiment, each DBL is labeled with a unique label, such that the the number of unique labels is equal to or greater than the number of bioactive agents (although in some cases, "reuse" of the unique labels can be done, as described herein; similarly, minor variants of candidate probes can share the same decoder, if the variants are

encoded in another dimension, i.e. in the bead size or label). For each bioactive agent or IBL, a DBL is made that will specifically bind to it and contains a unique label, for example one or more fluorochromes. Thus, the identity of each DBL, both its composition (i.e. its sequence when it is a nucleic acid) and its label, is known. Then, by adding the DBLs to the array containing the bioactive agents under conditions which allow the formation of complexes (termed hybridization complexes when the components are nucleic acids) between the DBLs and either the bioactive agents or the IBLs, the location of each DBL can be elucidated. This allows the identification of the location of each bioactive agent; the random array has been decoded. The DBLs can then be removed, if necessary, and the target sample applied.

In a preferred embodiment, the number of unique labels is less than the number of unique bioactive agents, and thus a sequential series of decoding steps are used. To facilitate the discussion, this embodiment is explained for nucleic acids, although other types of bioactive agents and DBLs are useful as well. In this embodiment, decoder probes are divided into **n** sets for decoding. The number of sets corresponds to the number of unique tags. Each decoder probe is labeled in **n** separate reactions with **n** distinct tags. All the decoder probes share the same **n** tags. Each pool of decoders contains only one of the **n** tag versions of each decoder, and no two decoder probes have the same sequence of tags across all the pools. The number of pools required for this to be true is determined by the number of decoder probes and the **n**. Hybridization of each pool to the array generates a signal at every address comprising an IBL. The sequential hybridization of each pool in turn will generate a unique, sequence-specific code for each candidate probe. This identifies the candidate probe at each address in the array. For example, if four tags are used, then 4 X **n** sequential hybridizations can ideally distinguish 4^n sequences, although in some cases more steps may be required. After the hybridization of each pool, the hybrids are denatured and the decoder probes removed, so that the probes are rendered single-stranded for the next hybridization (although it is also possible to hybridize limiting amounts of target so that the available probe is not saturated. Sequential hybridizations can be carried out and analyzed by subtracting pre-existing signal from the previous hybridization).

An example is illustrative. Assuming an array of 16 probe nucleic acids (numbers 1-16), and four unique tags (four different fluors, for example; labels A-D). Decoder probes 1-16 are made that correspond to the probes on the beads. The first step is to label decoder probes 1-4 with tag A, decoder probes 5-8 with tag B, decoder probes 9-12 with tag C, and decoder probes 13-16 with tag D. The probes are mixed and the pool is contacted with the array containing the beads with the attached candidate probes. The location of each tag (and thus each decoder and candidate probe pair) is then determined. The first set of decoder probes are then removed. A second set is added, but this time, decoder probes 1, 5, 9 and 13 are labeled with tag A, decoder probes 2, 6, 10 and 14 are labeled with tag B, decoder probes 3, 7, 11 and 15 are labeled with tag C, and decoder probes 4, 8, 12 and 16 are labeled with tag D. Thus, those beads that contained tag A in both decoding steps contain candidate probe 1; tag A in the first decoding step and tag B in the second decoding step contain candidate probe 2; tag A in the first decoding step and tag C in the second step contain candidate probe 3; etc.

As will be appreciated by those in the art, the decoder probes can be made in any order and added in any order.

In addition, since the size of the array will be set by the number of unique decoding binding ligands, there are a number of techniques that allow new combinations and the "reuse" of DBLs to allow for a greater number of test sites. This may be done in a wide variety of ways, as outlined below.

In a preferred embodiment, a spatial or positional coding system is done. In this embodiment, there are sub-bundles or subarrays (i.e. portions of the total array) that are utilized. By analogy with the telephone system, each subarray is an "area code", that can have the same labels (i.e. telephone numbers) of other subarrays, that are separated by virtue of the location of the subarray. Thus, for example, the same unique labels can be reused from bundle to bundle. Thus, the use of 50 unique labels in combination with 100 different subarrays can form an array of 5000 different bioactive agents. In this embodiment, it becomes important to be able to identify one bundle from another; in general, this is done either manually or through the use of marker beads; these can be beads containing unique tags for each subarray, or the use of the same marker bead in differing amounts, or the use of two or more marker beads in different ratios.

In a preferred embodiment, the coding and decoding is accomplished by sequential loading of the microspheres into the array. As outlined above for spatial coding, in this embodiment, the optical signatures can be "reused". In this embodiment, the library of microspheres each comprising a different bioactive agent (or the subpopulations each comprise a different bioactive agent), is divided into a plurality of sublibraries; for example, depending on the size of the desired array and the number of unique tags, 10 sublibraries each comprising roughly 10% of the total library may be made, with each sublibrary comprising roughly the same unique tags. Then, the first sublibrary is added to the fiber optic bundle comprising the wells, and the location of each bioactive agent is determined, generally through the use of DBLs. The second sublibrary is then added, and the location of each bioactive agent is again determined. The signal in this case will comprise the signal from the "first" DBL and the "second" DBL; by comparing the two matrices the location of each bead in each sublibrary can be determined. Similarly, adding the third, fourth, etc. sublibraries sequentially will allow the array to be filled.

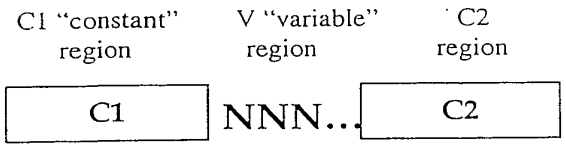
In a preferred embodiment, codes can be "shared" in several ways. In a first embodiment, a single code (i.e. IBL/DBL pair) can be assigned to two or more agents if the target analytes different sufficiently in their binding strengths. For example, two nucleic acid probes used in an mRNA quantitation assay can share the same code if the ranges of their hybridization signal intensities do not overlap. This can occur, for example, when one of the target sequences is always present at a much higher concentration than the other. Alternatively, the two target sequences might always be present at a similar concentration, but differ in hybridization efficiency.

Alternatively, a single code can be assigned to multiple agents if the agents are functionally equivalent. For example, if a set of oligonucleotide probes are designed with the common purpose of detecting the presence of a particular gene, then the probes are functionally equivalent, even though they may differ in sequence. Similarly, if classes or "families" of analytes are desired, all probes for different members of a class such as kinases or G-protein coupled receptors could share a code. Similarly, an array of this type could be used to detect homologs of known genes. In this embodiment, each gene is represented by a heterologous set of probes, hybridizing to different regions of the gene (and therefore differing in sequence). The set of probes share a common code. If a homolog is present, it might hybridize to some but not all of the probes. The level of homology might be indicated by the fraction of probes hybridizing, as well as the average hybridization intensity. Similarly, multiple antibodies to the same protein could all share the same code.

In a preferred embodiment, the set of codes can be increased using a kind of degeneracy on the IBLs on the beads. For most of the systems described herein, particularly when the IBL is a nucleic acid (including the case where the nucleic acid IBL is also the candidate agent), any single bead contains a single IBL sequence (decoding sequence). However, in this embodiment, it is possible to have a single bead comprise two or more decoding sequences, that differ at a particular nucleotide position. For a degeneracy of two at position X, there are 10 different possible combinations: the decoding sequence has an A at position X, a T, a C, a G, or a mixture of decoding sequences with either A/T, A/G, A/C, C/G, C/T or G/T. By using decoder probes that have different labels for each different nucleotide at the position, the number of codes can be increased, since beads containing the two different sequences will exhibit a mixture of signals. In addition, as will be appreciated by those in the art, rather than a "degeneracy" of two, degeneracies of three or four can be done as well. Thus, a bead containing three different decoding sequences will exhibit a signal comprising three different colors, etc.

Decoding the degenerate probes is done by a series of hybridizations to labeled oligonucleotides. Each hybridization decodes one position in the probe so the total number of hybridizations needed to decode a library is equal to the length of the variable region. Each hybridization is done with four oligonucleotides, each designed to hybridize specifically to a sequence containing a specific base at the interrogated position and each tagged with a distinctly colored fluorescent dye.

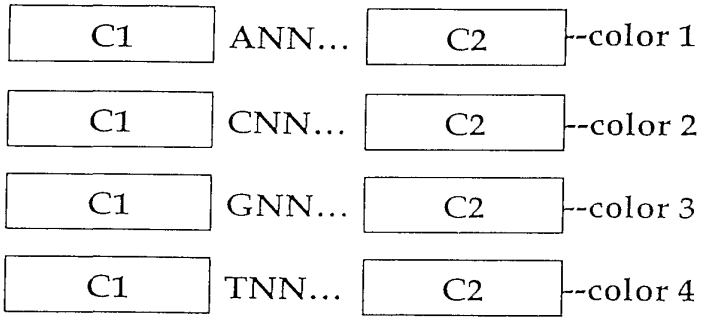
In a preferred embodiment, a degenerate probe sequence is attached to one or more constant probe regions. By "constant" probe region is meant a portion of a probe that is invariant among a population of decoder probes. In a particularly preferred embodiment, the degenerate or variable region is flanked on either side by constant regions. As such, each oligonucletoide has the following structure:



Although the C1 and C2 regions may be any length, preferably each is from 5 to 50 nucleotides in length. More preferably each is from 7 to 30 nucleotides in length and most preferably, each is about 10 bp in length. In one embodiment, the constant regions do not vary in sequence among the probes designed to decode a particular set of beads. As such, by using constant and variable regions (i.e. "CV" or "CVC" when two constant regions are used) in a set of probes, an array specific set or library of probes is generated.

The variable region contains a sequence that is unique to each address. Each "N" or variable position within the variable region can contain one of ten possibilities as described above. As such, the number of unique addresses in a library is determined by the length of the variable region: if L is length of the variable region, the number of unique addresses, A, is $A=10^{L}$.

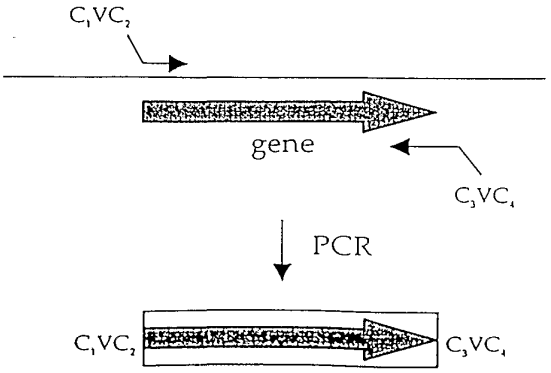
Accordingly, decoding the first base of the variable region of a decoding sequence is illustrated below:



Any address with an A at the first position would hybridize with the fourth oligonucleotide and give the corresponding color. An address with an A/C at the first position would hybridize with both the third and fourth decoders and give the corresponding combination of colors. By serial hybridizations, each using four decoders and each interrogating a different position of the V region, the entire sequence of each address can be determined. The "N' positions in the decoders are synthesized with degeneracies of the four standard bases.

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In one embodiment, CVC probes are incorporated into the probes of an oligonucleotide array during synthesis as is described for the addition of adapters; see U.S.S.Ns 09/553,993, filed April 20, 2000, 09/556,463, filed April 21, 2000, 60/160,917, filed October 22, 1999, and 60/135,123, filed May 20, 1999 and WO 00/63437, all of which are expressly incorporated herein by reference. In a similar fashion, the CVC probes could be synthesized in the 5-prime ends of PCR primers used to generate a PCR-product-based array. By incorporating different probes into the two primers of each PCR reaction, a combinatorial expansion of addresses is possible:



The constant regions in the C_1VC_2 addresses would be different in sequence from the C_3VC_4 addresses. Each would therefore be decoded with its own set of CVC complement decoders. If C_1VC_2 has a 3-base V region (1000 addresses) and C_3VC_4 has a 3-base V region (1,000 addresses), then the combination results in 1,000 x 1,000, or one million addresses.

In a preferred embodiment, there are additional ways to increase the number of unique or distinct tags. That is, the use of distinct attributes on each bead can be used to increase the number of codes. In addition, sequential decoding allows a reuse of codes in new ways. These attributes are independent of each other, thus allowing the number of codes to grow exponentially as a function of the number of decoding steps and the number of attributes (e.g. distinct codes). However, by increasing the amount of decoding information obtained in a single decoding step, the number of decoding steps is markedly reduced. By increasing the number of attributes per decoding step, fewer decoding steps are required for a given number of codes. Thus, in a preferred embodiment, a variety of methods are used to generate a number of codes for use in the process of decoding the arrays, while minimizing the necessary decoding steps. For example, a variety of different coding strategies can be combined: thus, different "colors", combinations of colors ("hues"), different intensities of colors or hues or both, different colors and different bead sizes, etc. can all be combined. Alternatively, the number of distinct codes is markedly increased.

In a preferred embodiment DBLs rely on attaching or embedding a quantitative or discrete set of physical attributes to the bead, i.e. labeling the bead. Preferred physical attributes of a bead include but are not limited to: surface "smoothness" or "roughness", color (fluorescent and otherwise), color

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intensity, size, detectable chemical moieties, chemical reactivity, magnetization, pH sensitivity, energy transfer efficiency between dyes present, hydrophobicity, hydrophilicity, absorptivity, charge, pH sensitivity, etc. In general, physical attributes that can be measured optically are preferred, including color, size, surface smoothness etc.

In a preferred embodiment, the DBLs may be reused by having some subpopulations of beads comprise optical signatures. In a preferred embodiment, the optical signature is generally a mixture of reporter dyes, preferably fluoroscent. By varying both the composition of the mixture (i.e. the ratio of one dye to another) and the concentration of the dye (leading to differences in signal intensity), matrices of unique optical signatures may be generated. This may be done by covalently attaching the dyes to the surface of the beads, or alternatively, by entrapping the dye within the bead. The dyes may be chromophores or phosphors but are preferably fluorescent dyes, which due to their strong signals provide a good signal-to-noise ratio for decoding. Suitable dyes for use in the invention include those listed for labeling DBLs, above.

In a preferred embodiment, the encoding can be accomplished in a ratio of at least two dyes, although more encoding dimensions may be added in the size of the beads, for example. In addition, the labels are distinguishable from one another; thus two different labels may comprise different molecules (i.e. two different fluors) or, alternatively, one label at two different concentrations or intensity.

In a preferred embodiment, the dyes are covalently attached to the surface of the beads. This may be done as is generally outlined for the attachment of the bioactive agents, using functional groups on the surface of the beads. As will be appreciated by those in the art, these attachments are done to minimize the effect on the dye.

In a preferred embodiment, the dyes are non-covalently associated with the beads, generally by entrapping the dyes in the pores of the beads.

Additionally, encoding in the ratios of the two or more dyes, rather than single dye concentrations, is preferred since it provides insensitivity to the intensity of light used to interrogate the reporter dye's signature and detector sensitivity.

In alternative embodiments, additional encoding parameters can be added, such as microsphere size. For example, the use of different size beads may also allow the reuse of sets of DBLs; that is, it is possible to use microspheres of different sizes to expand the encoding dimensions of the microspheres. Optical fiber arrays can be fabricated containing pixels with different fiber diameters or cross-sections; alternatively, two or more fiber optic bundles, each with different cross-sections of the individual fibers, can be added together to form a larger bundle; or, fiber optic bundles with fiber of the same size cross-sections can be used, but just with different sized beads. With different diameters, the largest wells can be filled with the largest microspheres and then moving onto progressively

smaller microspheres in the smaller wells until all size wells are then filled. In this manner, the same dye ratio could be used to encode microspheres of different sizes thereby expanding the number of different oligonucleotide sequences or chemical functionalities present in the array. Although outlined for fiber optic substrates, this as well as the other methods outlined herein can be used with other substrates and with other attachment modalities as well.

A bead decoding scheme includes assigning/imbuing a single quantifiable attribute to each bead type wherein each bead type differs in the quantifiable value of that attribute. For instance, one can attach a given number of fluorophores to a bead and quantitate the number of attached fluorophores in the decoding process; however, in practice, attaching a "given amount" of an attribute to a bead and accurately measuring the attribute may be problematic. Typically, the coefficient of variation (CV) seen in associating an attribute and the resultant measurement of this attribute is quite large (> 20%). By coefficient of variation is meant the variability in labeling a bead in successive labelings. This CV can be determined by labeling beads with a defined given number of label (fluorophore, for example) in multiple tests and measuring the resulting signal emitted by the bead. This large CV limits the number of useable and resolvable "levels" for any given attribute.

A more robust decoding scheme employs ratiometric rather than absolute measurements for segmenting a quantitative attribute into codes. By ratiometric decoding is meant labeling a bead with a ratio of labels or attributes (i.e. 1:10, 1:1, and 10:1). In theory any number of ratios can be used so long as the difference in signals between the ratios is detectable. This process produced smaller CVs and allowing more attribute segmentation within a given dynamic range. Thus, in a preferred embodiment, the use of ratiometric decoding reduces the coefficient of variability.

In addition, as will be appreciated by those in the art, ratiometric decoding can be accomplished in a different way. In this embodiment, rather than add a given number of beads with a first dye (or dye combination) intensity in the first decoding reaction and a second number with a second dye intensity in the sequential second decoding reaction, this ratiometric analysis may be done by using a ratio of labelled:unlabelled beads. That is, given a set saturating concentration of decoding beads, for example 100,000 beads/reaction, the first intensity decoding step may be done by adding 100,000 labelled beads and the second step can be done by adding 10,000 labelled beads and 90,000 unlabeled beads. Equilibrium dictates that the second step will give one tenth the signal intensity.

Because of the spread in values of a quantitatively measured attribute value, the number of distinct codes is practically limited to less than a dozen or so codes. However, by serially "painting" (i.e. temporarily attaching an attribute level to a bead) and "stripping" (removing the attribute level) a bead with different attribute values, the number of possible codes grows exponentially with the number of serial stages in the decoding process.

An example is illustrative. For instance, 9 different bead types and three distinguishable attribute distributions (Table 1). "Painting" (labeling) the beads with different attribute values in a combinatorially distinct pattern in the two different stages, generates a unique code for each bead type, i.e. nine distinct codes are generated. Thus, in a preferred embodiment beads are labeled with different attributes in a combinatorially distinct pattern in a plurality of stages. This generates unique codes for each bead type. Examples of different attributes are described above. Labeling of beads with different attributes is performed by methods known in the art.

	stage 1	stage 2	
Bead Type	attribute value	attribute value	Code
1	L	L	(L, L)
2	L	M	(L, M)
3	L	H	(L, H)
4	M	L	(M, L)
5	M	M	(M, M)
6	M	H	(M, H)
7	H	L	(H, L)
8	H	M	(H, M)
9	H	H	(H, H)

Number of unique codes =

Number of attributes$^{\text{Number of stages}}$

Table 1 Serial decode generates unique codes using a small number of attribute levels.

As outlined herein, fluorescent colors are a particularly convenient and preferable attribute to use in a decoding scheme. Fluorescent colors can be attached to any agent that recognizes an IBL to form a labeled DBL. A fluorescently labeled oligonucleotide is a particularly useful DBL since it can specifically and reversibly "paint" (label) any desired subset of beads with a particular color simply by the process of hybridization and dehybridization (i.e. to the DBL with a complementary sequence). Moreover, fluorescence is easily imaged and quantitated using standard optical hardware and software. In order to "paint" a given bead type with a particular color, the bead type must be labeled

with a unique hybridizable DNA sequence (IBL) and the decoding solution must contain the color-labeled complement of that sequence.

One consideration in implementing a decoding scheme is to minimize the number of images collected. In a color-based scheme, the number of images collected is the product of the number of colors and the number of stages. The number of images can be reduced by "painting" a bead with multiple colors for each given stage. By assigning multiple colors to a bead, the number of effective codes is increased. As an example, in a 24 bit three color scheme (e.g. red, green, blue) coloring process used by computers, a total of 256*256*256 = 16.7 million different "hues" can be generated from just three colors (red, green, blue).

Thus, in a preferred embodiment DBLs are labeled with a combination of colored fluorophores. As such, this method finds use in increasing the number of available codes for labeling DBLs using only a handful of different dyes (colors). Increasing the number of codes available at each decoding step will greatly decrease the number of decoding steps required in a given decoding process.

In one embodiment a population of oligonucleotides encoding a single DBL is labeled with a defined ratio of colors such that each bead to which the DBL binds is identified based on a characteristic "hue" formulated from the combination of the colored fluorophores. In a preferred embodiment two distinct colors are used. In a preferred embodiment, three or more distinct dyes (colors) are available for use. In this instance the number of differentiable codes generated by labeling a population of oligonucleotides encoding a single DBL with any given color is three. However by allowing combinations of colors and color levels in the labeling, many more codes are generated.

For decoding by hybridization, a preferred number of distinguishable color shades is from 2 to 2000; a more preferred number of distinguishable color shades is from 2 to 200 and a most preferred number of distinguishable color shades is from 2 to 20. Utilizing three different color shades (intensities) and three colors, the number of different hues will be $3^4 = 81$. Combining a hue with sequential decoding allows a virtually limitless number of codes to be generated.

As previously described, the DBL can be any agent that binds to the IBL. In a preferred embodiment, a single DBL is labeled with a pre-determined ratio of colors. This ratio is varied for each DBL thus allowing for a unique "hue" for each DBL labeled as such. Following treatment of the beads with the DBL, the bead is analyzed to determine the "hue" associated with each bead, thereby identifying the bead with its associated bioactive agent.

For instance, with four primary colors and two intensity levels (color is present or absent), fifteen different hues/stage are possible. If four dyes and three different intensity levels are used (absent, half-present, fully present), then 73 different hues/stage are possible. In this case, acquisition of only 4 color images is sufficient to obtain information on 73 different coding hues.

In a preferred embodiment the DBL relies on a Fluorescence Resonance Energy Transfer (FRET) identifier. Fluorescence resonance energy transfer (FRET) is well known in the art and has been employed for various biotechnological applications. FRET requires that the energy of the donor excited state overlap with the ground state of the acceptor, implying that the donor emission spectrum overlaps with the acceptor absorbance spectrum. In a FRET experiment, the donor is excited with light; some of the energy of the excited state is released as light emission while some of the energy is transferred to the acceptor, which becomes excited emitting light at a characteristic wavelength. The distance over which energy transfer may occur can be varied by the choice of energy donor and acceptor, wherein the use of various different donor and acceptor molecules is contemplated. The formula for the efficiency of energy transfer is:

$$T = R_0^6/(R_0^6+R^6)$$

Where R is the distance between the donor and acceptor, and R_0 is a constant related to the characteristic overlap of the absorption and emission spectra of the donor-acceptor pair.

A FRET identifier comprises a linker region that is labeled at each terminus with a fluorophore. The first fluorophore serves as a donor fluorophore, whose signal is detectable; the other acts as an acceptor fluorophore. The emission of the acceptor fluorophore also is a detectable signal, the intensity of which varies proportionally with the distance between the two fluorophores. That is, by varying the distance between the two fluorophores different codes can be generated.

Identification of the decoding oligonucleotide, therefore, is accomplished by identifying the "efficiency" or ratio of intensities of the acceptor fluorophore and the donor fluorophore. This ratio should be invariant with regard to the number of decoding oligonucleotides bound to a particle (bead); rather, without being bound by any theory, the distance between the fluorophores determines the ratio. Thus, by varying the distance between the fluorophores (i.e. increasing or decreasing the length of the linker), unique "codes" can be assigned to the DBLs. Each code is characterized by the ratio of emission intensities between the two fluorophores.

An even greater advantage is realized when different bead attributes can be collected in a single image rather than from multiple images. For instance, if the colors of red, green and blue are used as attributes, three separate images need to be collected. However, if different shades or intensities of each color are used in the coding process, a much larger number of attributes can be realized in a single image. The difficulty with using mixtures of colors, is that the color ratios can vary somewhat between bead types since individual components are mixed together to determine the color ratio. If on the other hand, the color ratio is built into the decoding label , as with the above described FRET decoding oligonucleotides, the decoding process should be more reliable and more efficient.

In preferred embodiments of the present invention, the first component may be a lanthanide such as a chelate of europium(III) (Eu(III)) or terbium(III) (Tb(III)) which serves as a fluorescent donor and the second component may be an activated ester of cyanine 5 (Cy5) or tetramethylrhodamine (TMR) which serve as energy acceptors of Eu(III) and Tb(III). Other first and second component pairs that will find use for FRET include, for example, fluorescein and rhodamine; FITC and rhodamine; and fluorescein and trinitrophenyl, and other components as are well known in the art.

The linker is comprised of any spacer element including alkyl linkers, polymer linkers, etc. For ease of synthesis, preferably the linker comprises nucleic acids (including nucleic acid analogs).

The FRET decoding oligonucleotide comprises a spacer or linker region that is labeled with fluorophores (dyes). In general, this may be used in two general ways. In a preferred embodiment, as generally depicted in Figure 4, the linker is attached to the terminus of a decoding nucleotide that hybridizes with (is complementary to) the IBL. In this embodiment, the linker length can be varied to adjust signal intensity. Thus, in a preferred embodiment a FRET oligonucleotide is adjoined to an oligonucleotide DBL. In one embodiment the FRET oligonucleotide is synthesized independent of the DBL oligo. Subsequently the FRET oligonucleotide is ligated to the DBL oligo according to methods well known in the art. Alternatively, the DBL oligo and FRET oligo are synthesized as a single oligonucleotide comprising the FRET fluorophores at the desired positions.

Alternatively, in a second embodiment the linker is itself the oligonucleotide that hybridizes with (is complementary to) the IBL. That is, fluorophores are attached to the decoding oligonucleotide at different basepair lengths; some may be 10 nucleotides apart, some 20, etc. In a preferred embodiment the oligonucleotide DBL is itself labeled with fluors; as such the oligonucleotide serves as both DBL and FRET oligonucleotide.

The linker region of a FRET oligonucleotide comprises any set of nucleotides; in a preferred embodiment, for example when the linker does not hybridize to anything, the linker comprises a single repeated nucleotide. A single repeated nucleotide is less likely to hybridize with the IBL or bioactive agent on the bead because of the reduced likelihood of the presence of a complementary nucleotide sequence in either the IBL or bioagent on the bead. In a preferred embodiment, the linker comprises a polynucleotide linker such as poly T, poly A, poly G or poly C, or combinations.

The linker can be of any length so long as the emission of the first fluorophore is absorbed by the second fluorophore. In a preferred embodiment the linker is formed of from 1 to at least 100 nucleotides. More preferably the linker is formed from 1 to 70 nucleotides. Most preferably the linker is formed from 1 to 40 nucleotides, with from 5 to 20 being especially preferred.

In a preferred embodiment, for a given DBL sequence, several codes can be generated by varying the distance of separation of the two fluorophores and hence generating several energy transfer "efficiency" ratios.

In another preferred embodiment, for a given DBL sequence, a single code can be generated by varying the distance of separation of the two flourophores and hence generating a single energy transfer ratio for a single DBL. However, numerous DBLs can be labeled with distinct FRET oligos thus allowing for the simultaneous determination or identification of multiple DBLs in a single imaging session as a result of the plurality of energy transfer ratios.

For example, given a 40-mer decoding oligo, the donor and acceptor fluorophores can be located as close as one or as far apart as 40 bases. In principle this should allow 39 codes to be generated for a single decoding sequence. Empirically, the number of codes generated depends on dispersion in the distributions for the ratiometric measurements.

Also, given a 40-mer decoding oligo, 39 unique codes can be generated which will identify 39 different oligos.

The present invention provides array compositions comprising a substrate with a surface comprising discrete sites. A population of microspheres is distributed on the sites, and the population comprises at least a first and a second subpopulation. Each subpopulation comprises a bioactive agent, and, in addition, at least one optical dye with a given pKa. The pKas of the different optical dyes are different.

In a preferred embodiment, when for example the array comprises cloned nucleic acids, there are several methods that can be used to decode the arrays. In a preferred embodiment, when some sequence information about the cloned nucleic acids is known, specific decoding probes can be made as is generally outlined herein.

In a preferred embodiment, "random" decoding probes can be made. By sequential hybridizations or the use of multiple labels, as is outlined above, a unique hybridization pattern can be generated for each sensor element. This allows all the beads representing a given clone to be identified as belonging to the same group. In general, this is done by using random or partially degenerate decoding probes, that bind in a sequence-dependent but not highly sequence-specific manner. The process can be repeated a number of times, each time using a different labeling entity, to generate a different pattern of signals based on quasi-specific interactions. In this way, a unique optical signature is eventually built up for each sensor element. By applying pattern recognition or clustering algorithms to the optical signatures, the beads can be grouped into sets that share the same signature (i.e. carry the same probes).

In order to identify the actual sequence of the clone itself, additional procedures are required; for example, direct sequencing can be done. By using an ordered array containing the clones, such as a spotted cDNA array, a "key" can be generated that links a hybridization pattern to a specific clone whose position in the set is known. In this way the clone can be recovered and further characterized.

Alternatively, clonal arrays can be decoded using binary decoding with vector tags. For example, partially randomized oligos are cloned into a nucleic acid vector (e.g. plasmid, phage, etc.). Each oligonucleotide sequence consists of a subset of a limited set of sequences. For example, if the limites set comprises 10 sequences, each oligonucleotide may have some subset (or all of the 10) sequences. Thus each of the 10 sequences can be present or absent in the oligonucleotide. Therefore, there are 2^{10} or 1,024 possible combinations. The sequences may overlap, and minor variants can also be represented (e.g. A, C, T and G substitutions) to increase the number of possible combinations. A nucleic acid library is cloned into a vector containing the random code sequences. Alternatively, other methods such as PCR can be used to add the tags. In this way it is possible to use a small number of oligo decoding probes to decode an array of clones.

There are a variety of methods used to evaluate decoding. In a preferred embodiment, discriminant analysis and cluster algorithms and computer apparatus are used to analyze the decoding data from the arrays of the invention. The potentially large number of codes utilized in the invention, coupled with the use of different intensities and "hues" of fluorophores in multi-step decoding processes, requires good classification of the data. The data, particularly intensity data, is acquired in a multi-step process during which beads are "painted" (for example by hybridizing dye-labeled complementary decoding oligonucleotides to the IBL probes on the beads, or the formation of binding ligand pairs for non-nucleic acid IBL-DBL pairs) with different colors or mixtures of colors ("hues") at each stage. The challenge is to accurately classify a bead as to which color with which it was painted at each step. The more closely related the "paints" (colors) are to one another (as determined by the optical imaging system), the more difficult the classification.

The proximity of the dyes as seen by the imaging system is determined by the spectral properties of the decoding dyes and the spectral channel separation of the imaging system. Better color separation is achieved by employing fluorescent dyes with narrow emission spectra, and by employing an optical system with narrow band pass excitation and emission filters which are designed to excite the dye "on peak" and measure its emission "on peak". The process of optically imaging the dyes on the beads is similar to the human vision process in which our brain sees color by measuring the ratio of excitation in the three different cone types within our eye. However, with an optical imaging system, the number of practical color channels is much greater than the three present in the human eye. CCD based imaging systems can "see" color from 350 nm up to 850 nm whereas the cones in the eye are tuned to the visible spectrum from 500 - 600 nm.

The problem of decoding bead arrays is essentially a discriminant analysis classification problem. Thus, in a preferred embodiment, an analysis of variance in hyperspectral alpha space is performed on a known set of bead colors or hues. The center of the bead clusters in alpha space are termed the centroids of the clusters, and the scatter of the points within a cluster determines the spread of the cluster. A robust classification scheme requires that the distance between the centroids of the different bead classes (hues) is much greater than the spread of any cluster class. Moreover, the location of the centroids should remain invariant from substrate to substrate and from experiment to experiment.

Thus, in a preferred embodiment, a hue "zone" is defined as a region in alpha space surrounding the hue centroid and extending out to the spread radius of the cluster. Given a reference set of hue centroids and spread radii, as determined empirically, the classification of a new set of data can be accomplished by asking whether a given bead point falls closest to or within the "zone" of a hue cluster. This is accomplished by calculating the Mahalanobis distance (in this case, it is simply a Euclidean distance metric) of the bead point from the centroids of the different hue classes. For the data shown in Fig. 6, the location of the centroids and their distances from one another are indicated in Table 2.

Table 2	Centroid position				Distance between centroids			
dye/channel	Blue	Green	Yellow	Red	Bod-493	Bod-R6G	Bod-564	Bod-TXR
Bod-493	0.63	0.22	0.11	0.03	0.00			
Bod-R6G	0.03	0.51	0.37	0.09	0.72	0.00		
Bod-564	0.06	0.04	0.57	0.32	0.81	0.55	0.00	
Bod-TXR	0.09	0.05	0.04	0.82	0.99	0.93	0.73	0.00

For classifying the different beads into a particular hue class, a Euclidean distance cutoff of 0.3 was chosen. The closest two centroids, the Bod-R6G and Bod-564 (dist = 0.55), have a slight overlap in their decoding zones when using a Euclidean or Mahalanobis distance of 0.3. An improvement in classification can be achieved by decreasing this distance, and by weighting the different coordinate axes appropriately.

Accordingly, the present invention provides computer methods for analyzing and classifying the color of a bead. The classification of the color of the bead is done by viewing the bead in hyperspectral "alpha" space ($\Delta_1 = I_1/6I_I$, $\Delta_2 = I_2/6I_i$, $\Delta_3 = I_3/6I_i$,, etc.) in which each coordinate axis represents the fraction of the bead intensity within a given imaging channel. For instance, if four imaging channels are used to image the beads, the color or hue of a bead can be represented by a point in 3-D alpha space (the fourth dimension is not necessary since $Sa_i = 1$). Given a set of different primary dyes by which to "paint" the beads, the number of hues that can be generated from these dyes is unlimited since the dyes can be combined in varying ratios and in varying combinatorial patterns. The

number of practical hues is experimentally determined by the separation of the different hue clusters in hyperspectral alpha space.

Fig. 6 shows a hyperspectral alpha plot of beads "painted" with four different hues imaged in four separate imaging channels. Note that the beads form four distinct clusters. The fact that these four clusters are well separated allows a robust decode classification scheme to be implemented.

In a preferred embodiment, a quality control analysis of the decoding process is done. This is achieved by performing a cluster analysis of alpha space for each decoding stage. The number of clusters determined will be fixed by the expected number of hues. The positions of the cluster centroids will be monitored and any deviations from the expected position will be noted.

Thus the invention provides an apparatus for decoding the arrays of the invention. In addition to the compositions outlined herein, the apparatus includes a central processing unit which communicates with a memory and a set of input/output devices (e.g., keyboard, mouse, monitor, printer, etc.) through a bus. The general interaction between a central processing unit, a memory, input/output devices, and a bus is known in the art. One aspect of the present invention is directed toward the hyperspectral "alpha" space classification system stored in the memory.

The classification system program includes a data acquisition module that receives data from the optical reader or confocal microscope (or other imaging system). In general, the classification program also includes an analysis module, that can analyze the variance in hyperspectral alpha space, calculate the centroids of the clusters, calculate the scatter of the cluster (the spread) and define the hue zone and distance cutoff. In general, the analysis module will further determine whether a data point falls within the hue zone by calculating the Mahalanobis distance.

Finally, the analysis module will analyze the different sequential decoding information to finally assign a bioactive agent to a bead location.

In this way, sequential decoding steps are run, with each step utilizing the discriminant analysis calculations to assign each bead in the array to a hue cluster at each step. The buildup of the sequential decoding information allows the correlation of the location of a bead and the chemistry contained on it.

In one embodiment, the invention includes modifying the number of detection channels available for analysis relative to the number of labels to be detected. That is, in one embodiment, the number of detection channels is decreased relative to the number of labels used. In an alternative embodiment the number of detection channels is increased relative to the number of labels used.

When fewer detection channels are used, fewer images are required during decoding. As such the invention provides a method of reducing the number of images obtained during decoding. Fluorescent dyes have broad band absorption and emission spectra. Accordingly, the emission of certain dyes is detected in multiple channels, generally by choosing channels to maximize the signal. However by choosing the dyes and the channels carefully, fewer channels that dyes may be used. As such, decoding with fewer channels than dyes, allows for detection of signals that are maximally detected in a particular channel, and overlapping in several channels. Thus, for example, beads with dye-1 are read in channel-1, beads with dye-2 are read in channel-2 and beads with dye-3 show signals in both channel-1 and channel-2. Example 7 is illustrative and the results of the experiment are found in Figure 11. It is noted, however, that three dyes are clearly detected and distinguished following detection in only two channels. Accordingly, using fewer detection channels than the number of dyes to be analyzed, allows for a reduction in the number of images and steps required for decoding.

In an alternative embodiment, the invention provides for using additional channels to increase the number of detectable codes. That is, additional readout channels are used to increase the coding/decoding capacity of arrays by analyzing attributes of the bead in addition to an optical signal such as fluorescence. For example, bead size, shape, density and surface characteristics can alter the light-scattering properties of the beads and can be detected optically. Additional channels that provide information independent of fluorescence are forward-scatter and side-scatter. Low-angle forward light scatter provides some information on the relative size of beads, whereas side light scatter provides some information on the relative granularity or texture of individual beads. By analogy these channels are often used in combination to distinguish the different major categories of white cells in unseparated mammalian blood, but are useful in a wide variety of other assays as well.

With respect to particle size, particles may simply be varied in diameter. Alternatively, particles could be assembled as doublets, triplets, or other higher-order multimers. Doublets and triplets often occur spontaneously in a population, and can be enriched for by fractionation.

With respect to surface properties, in one embodiment, the invention provides the use of metallization. This can dramatically alter the scattering properties of a surface. One way to accomplish this is to attach gold particles (e.g. colloidal gold) to an affinity molecule, such as streptavidin or DNA, and then to bind the molecule at defined loadings to specific sites on the bead. For example, ten different loadings could give ten new codes. Together with 100 fluorescence codes, this would allow the recognition of ~ 1000 bead types in an array. Alternatively, metallization could be used for the assay readout, and fluorescence for the encoding.

With respect to magnetism, superparamagnetic beads could be deflected according to the amount of magnetism, and directed to different readout channels -i.e. this mechanism could be used as a

"pre-sorter" prior to fluorescence readout. In one version, bead subpopulations could be localized to different regions of a reaction vessel (e.g. microtiter well) by the use of small magnets. Each sub-population could be released sequentially for analysis.

Although the previously described method involving additional detection channels finds use in planar bead arrays, it also finds particular use in liquid arrays. By "liquid arrays" is meant a collection of beads that are not spatially organized, but are typically encoded by fluorescence labeling and "decoded" by readout on a flow- cytometer or equivalent instrument. Assays are carried out in bulk in solution. Generally, liquid arrays are designed such that the assay result and decoding result are obtained essentially simultaneously. However, because only optical images are detected simultaneously, the number of codes that can be analyzed is limited. That is, there is a limit to the number of codes that can be used since the number of assay signals and encoding signals must be determined at different wavelengths at essentially the same time. However, by using additional detection channels as described above, the number of codes that can be analyzed is markedly increased.

This method finds particular use in analyzing the results of genomics based assays such as gene expression profiling, genotyping and SNP analysis. In such genomics based assays, it is frequreintly desirable to make hundreds to millions of measurements simultaneously. Thus, by increasing the number of codes that can be simultaneously detected, analysis of such assays is enhanced.

This invention provides methods of maximizing the information that is obtained from a single bead. That is, by providing ways of multiplexing assays on a single bead, multiple parameters can be simultaneously detected. That is, after a cycle of performing a particular assay, decoding and reading the assay results, the bead population is recovered. The detection molecules are removed from the bead and the cycle is repeated.

In a preferred embodiment, parity analysis is used during decoding to increase the robustness and accuracy of the system. By "parity analysis"is meant a decoding step wherein the signal of a particular element is analyzed across a plurality of decoding stages. That is, following at least one decoding step, but most preferably following multiple decoding steps, the signal of an array element across the decoding stages is analyzed. That is, the signal from a particular bead is evaluated across multiple stages. Although the analysis includes any parameter that can be obtained from the signals such as evaluating the total signal obtained across the stages, in a preferred embodiment, the parity of the signals across the stages is analyzed.

By "parity" is meant the digital or modular readout of signals, i.e. odd or even, when binary signals are used. As depicted in Figure 7, the digit sum of the signals across a plurality of stages is translated into a parity determination. The parity determination is then useful in evaluating the decoding process. An

example is illustrative. Codes are designed to have an odd number of a particular signal, for example a red signal, when viewed across all stages or decoding steps, or a pre-determined plurality of stages or decoding steps. The detection of an even number of red stages, provides an indication that an error has occurred at some point in decoding. When this result is obtained, the faulty code can either be discarded, or the analysis repeated.

In a preferred embodiment the invention includes introducing a "redundant stage" into the decoding system. By "redundant stage" is meant a stage that serves as a parity check. That is, following the decoding stages, an additional stage is included to analyze the parity. This analysis provides an indication of the competence or validity of the decoding. That is, when codes are designed with a pre-determined parity, the redundant stage is used to detect the parity of the signals obtained from the decoding step. That is, the redundant stage detects errors in parity because if there has been an error in decoding, the parity detected following the redundant stage will be different from the parity designed into the codes.

An example is illustrative. In the following illustration, array elements are decoded in two stages with two colors. This results in the detection of 4 (2^2) codes. The codes are designed to result in odd parity following the analysis with the redundant stage. That is, the code sequences can be made such that each code will contain an odd number of "red" colors (when viewed across all stages, or when viewed across the pre-determined number of stages). In the illustration below, only the stage where the "red" signal is detected is indicated.

Code	Stage 1	Stage 2	Redundant Stage	Parity (number of "red" stages)
1	0	0	red	1 (odd)
2	0	red	0	1 (odd)
3	red	0	0	1 (odd)
4	red	red	red	3 (odd)

However, if there is an error during decoding, this will be reflected as detecting a parity that is different from that designed into the codes. In the following illustration the same codes as above are used however, an error is detected. In this case if one detects an even number of "red" stages it can be inferred that there has been a decoding error.

Code	Stage 1	Stage 2	Redundant Stage	Parity (number of "red" stages)
1	0	0	red	1 (odd)
2	0	red	0	1 (odd)

3	red	0	0	1 (odd)
4	red	0 (error)	red	2 (even)

Although in some embodiments, the parity is binary, i.e. odd or even, in other embodiments the parity is modular or based on numbers other than 2. That is, the parity can be based on detection of 3 or more signals. In a preferred embodiment, the signals are multiple colors. In a particularly preferred embodiment the colors are red, green and blue.

An example is illustrative. In the following illustration, codes are decoded in two steps with three colors. This results in the detection of 3^2 (9) signals. In the illustration, red is indicated by 1, green is indicated by 2 and blue is indicated by 3. The parity is based on a modulus 3. By including a third stage as a redundant stage the parity can be checked as described above.

Code	Stage 1	Stage 2	Redundant stage	Parity
1	1	1	1	3
2	1	2	3	6
3	1	3	2	6
4	2	1	3	6
5	2	2	2	6
6	2	3	1	6
7	3	1	2	6
8	3	2	1	6
9	3	3	3	9

However, when there is a decoding error, the parity will be incorrect; in the illustration the parity readout is not divisible by 3.

Code	Stage 1	Stage 2	Redundant stage	Parity
1	1	1	1	3
2	1	2	3	6
3	1	3	2	6
4	2	1	3	6
5	2	2	2	6

6	2	3	1	6
7	3	1	2	6
8	3	2	1	6
9	3	2 (error)	3	8

Thus, a redundant stage allows for the detection of errors on the array or in the signal obtained from an array.

Although described above for detecting errors on arrays, the method of using redundant stages is not limited to arrays. That is, the method finds use in detecting errors in a variety of systems that send, receive and/or interpret or analyze data such as communications systems including modems etc. In particular, the method of using the modular redundant stages allows for processing of signals of vastly increased complexity as compared to sending bits of data. That is, when using modular redundant stages, characters as opposed to bits, can be sent across modem lines in the communications field. The modular redundant stage is used to detect errors in the communication or processing of the signals.

In an additional embodiment a redundant channel is used during the decoding. By "redundant channel" is meant either the use of multiple filters to analyze a particular signal or repeated use of the same filter to analyze the signal, i.e. multiple signals from the same channel. Redundant channels find use as a source of a "back-up" signal of sorts for a particular array element. That is, when multiple images are obtained as a result of redundant channels, the likelihood of obtaining the correct signal increases. That is, when it is realized that one of the results for an element is compromised for some reason, the image obtained from the redundant channel is used instead. When the initial image is not compromised, the result from the redundant channel need not be used, although it may be used, for example as a way to confirm the initial signal.

An example is illustrative. During decoding, when it is apparent that one stage fails, for example as a result of mis-registration of a channel, the use of a redundant channel serves to rescue the decoding at that particular stage. Mis-registration can also be addressed as outlined in USSN 09/636,387, filed August 9, 2000, which is expressly incorporated herein by reference. As noted above, the redundant channel can either be an image separate filter to analyze the signal, or alternatively, it can be multiple images taken from the same filter.

In one embodiments, the number of redundant channels increases as the number of decoding stages increases. Because the investment in decoding increases as each decoding stage or step proceeds, it is important in this embodiment to ensure the competence of decoding at later decoding stages.

In a preferred embodiment, redundant systems are used to avoid the loss of information. It is appreciated that when "S" decoding steps (sometimes referred to herein as "stages") and "C" colors are used, the maximum number of distinct signals is C^S (C raised to the "S"th power). In some instances, the corruption of a stage results in the loss of C^S- (C-1)x$C^{(S-1)}$ codes. Depending on the values of C and S, the loss can be substantial. Accordingly, the present invention provides for methods of improving the robustness of the decoding system. That is, the invention provides for a method of confirming the results of a decoding analysis or detecting errors in a decoding analysis during or after the decoding procedure. The method also decreases the number of false positive or false negative signals; similarly, the number of true positives and/or true negatives increases.

In a preferred embodiment, several levels of redundancy are built into the arrays of the invention. Building redundancy into an array gives several significant advantages, including the ability to make quantitative estimates of confidence about the data and signficant increases in sensitivity. Thus, preferred embodiments utilize array redundancy. As will be appreciated by those in the art, there are at least two types of redundancy that can be built into an array: the use of multiple identical sensor elements (termed herein "sensor redundancy"), and the use of multiple sensor elements directed to the same target analyte, but comprising different chemical functionalities (termed herein "target redundancy"). For example, for the detection of nucleic acids, sensor redundancy utilizes of a plurality of sensor elements such as beads comprising identical binding ligands such as probes. Target redundancy utilizes sensor elements with different probes to the same target: one probe may span the first 25 bases of the target, a second probe may span the second 25 bases of the target, etc. By building in either or both of these types of redundancy into an array, significant benefits are obtained. For example, a variety of statistical mathematical analyses may be done.

In addition, while this is generally described herein for bead arrays, as will be appreciated by those in the art, this techniques can be used for any type of arrays designed to detect target analytes.

In a preferred embodiment, sensor redundancy is used. In this embodiment, a plurality of sensor elements, e.g. beads, comprising identical bioactive agents are used. That is, each subpopulation comprises a plurality of beads comprising identical bioactive agents (e.g. binding ligands). By using a number of identical sensor elements for a given array, the optical signal from each sensor element can be combined and any number of statistical analyses run, as outlined below. This can be done for a variety of reasons. For example, in time varying measurements, redundancy can significantly reduce the noise in the system. For non-time based measurements, redundancy can significantly increase the confidence of the data.

In a preferred embodiment, a plurality of identical sensor elements are used. As will be appreciated by those in the art, the number of identical sensor elements will vary with the application and use of the sensor array. In general, anywhere from 2 to thousands may be used, with from 2 to 100 being preferred, 2 to 50 being particularly preferred and from 5 to 20 being especially preferred. In general,

preliminary results indicate that roughly 10 beads gives a sufficient advantage, although for some applications, more identical sensor elements can be used.

Once obtained, the optical response signals from a plurality of sensor beads within each bead subpopulation can be manipulated and analyzed in a wide variety of ways, including baseline adjustment, averaging, standard deviation analysis, distribution and cluster analysis, confidence interval analysis, mean testing, etc.

In a preferred embodiment, the first manipulation of the optical response signals is an optional baseline adjustment. In a typical procedure, the standardized optical responses are adjusted to start at a value of 0.0 by subtracting the integer 1.0 from all data points. Doing this allows the baseline-loop data to remain at zero even when summed together and the random response signal noise is canceled out. When the sample is a fluid, the fluid pulse-loop temporal region, however, frequently exhibits a characteristic change in response, either positive, negative or neutral, prior to the sample pulse and often requires a baseline adjustment to overcome noise associated with drift in the first few data points due to charge buildup in the CCD camera. If no drift is present, typically the baseline from the first data point for each bead sensor is subtracted from all the response data for the same bead. If drift is observed, the average baseline from the first ten data points for each bead sensor is substracted from the all the response data for the same bead. By applying this baseline adjustment, when multiple bead responses are added together they can be amplified while the baseline remains at zero. Since all beads respond at the same time to the sample (e.g. the sample pulse), they all see the pulse at the exact same time and there is no registering or adjusting needed for overlaying their responses. In addition, other types of baseline adjustment may be done, depending on the requirements and output of the system used.

Once the baseline has been adjusted, a number of possible statistical analyses may be run to generate known statistical parameters. Analyses based on redundancy are known and generally described in texts such as Freund and Walpole, Mathematical Statistics, Prentice Hall, Inc. New Jersey, 1980, hereby incorporated by reference in its entirety.

In a preferred embodiment, signal summing is done by simply adding the intensity values of all responses at each time point, generating a new temporal response comprised of the sum of all bead responses. These values can be baseline-adjusted or raw. As for all the analyses described herein, signal summing can be performed in real time or during post-data acquisition data reduction and analysis. In one embodiment, signal summing is performed with a commercial spreadsheet program (Excel, Microsoft, Redmond, WA) after optical response data is collected.

In a preferred embodiment, cummulative response data is generated by simply adding all data points in successive time intervals. This final column, comprised of the sum of all data points at a particular

time interval, may then be compared or plotted with the individual bead responses to determine the extent of signal enhancement or improved signal-to-noise ratios.

In a preferred embodiment, the mean of the subpopulation (i.e. the plurality of identical beads) is determined, using the well known Equation 1:

Equation 1

$$\mu = \sum \frac{x_i}{n}$$

In some embodiments, the subpopulation may be redefined to exclude some beads if necessary (for example for obvious outliers, as discussed below).

In a preferred embodiment, the standard deviation of the subpopulation can be determined, generally using Equation 2 (for the entire subpopulation) and Equation 3 (for less than the entire subpopulation):

Equation 2

$$\sigma = \sqrt{\frac{\sum (x_i - \mu)^2}{n}}$$

Equation 3

$$s = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}}$$

As for the mean, the subpopulation may be redefined to exclude some beads if necessary (for example for obvious outliers, as discussed below).

In a preferred embodiment, statistical analyses are done to evaluate whether a particular data point has statistical validity within a subpopulation by using techniques including, but not limited to, t distribution and cluster analysis. This may be done to statistically discard outliers that may otherwise skew the result and increase the signal-to-noise ratio of any particular experiment. This may be done using Equation 4:

Equation 4

$$t = \frac{\bar{x} - \mu}{s/\sqrt{n}}$$

In a preferred embodiment, the quality of the data is evaluated using confidence intervals, as is known in the art. Confidence intervals can be used to facilitate more comprehensive data processing to measure the statistical validity of a result.

In a preferred embodiment, statistical parameters of a subpopulation of beads are used to do hypothesis testing. One application is tests concerning means, also called mean testing. In this application, statistical evaluation is done to determine whether two subpopulations are different. For example, one sample could be compared with another sample for each subpopulation within an array to determine if the variation is statistically significant.

In addition, mean testing can also be used to differentiate two different assays that share the same code. If the two assays give results that are statistically distinct from each other, then the subpopulations that share a common code can be distinguished from each other on the basis of the assay and the mean test, shown below in Equation 5:

Equation 5

$$z = \frac{\overline{x_1} - \overline{x_2}}{\sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}}$$

Furthermore, analyzing the distribution of individual members of a subpopulation of sensor elements may be done. For example, a subpopulation distribution can be evaluated to determine whether the distribution is binomial, Poisson, hypergeometric, etc.

In addition to the sensor redundancy, a preferred embodiment utilizes a plurality of sensor elements that are directed to a single target analyte but yet are not identical. For example, a single target nucleic acid analyte may have two or more sensor elements each comprising a different probe. This adds a level of confidence as non-specific binding interactions can be statistically minimized. When nucleic acid target analytes are to be evaluated, the redundant nucleic acid probes may be overlapping, adjacent, or spatially separated. However, it is preferred that two probes do not compete for a single binding site, so adjacent or separated probes are preferred. Similarly, when proteinaceous target analytes are to be evaluated, preferred embodiments utilize bioactive agent binding agents that bind to different parts of the target. For example, when antibodies (or antibody fragments) are used as bioactive agents for the binding of target proteins, preferred embodiments utilize antibodies to different epitopes.

In this embodiment, a plurality of different sensor elements may be used, with from about 2 to about 20 being preferred, and from about 2 to about 10 being especially preferred, and from 2 to about 5

being particularly preferred, including 2, 3, 4 or 5. However, as above, more may also be used, depending on the application.

As above, any number of statistical analyses may be run on the data from target redundant sensors.

One benefit of the sensor element summing (referred to herein as "bead summing" when beads are used), is the increase in sensitivity that can occur.

Once made, the compositions of the invention find use in a number of applications. In a preferred embodiment, the compositions are used to probe a sample solution for the presence or absence of a target analyte, including the quantification of the amount of target analyte present. By "target analyte" or "analyte" or grammatical equivalents herein is meant any atom, molecule, ion, molecular ion, compound or particle to be either detected or evaluated for binding partners. As will be appreciated by those in the art, a large number of analytes may be used in the present invention; basically, any target analyte can be used which binds a bioactive agent or for which a binding partner (i.e. drug candidate) is sought.

Suitable analytes include organic and inorganic molecules, including biomolecules. When detection of a target analyte is done, suitable target analytes include, but are not limited to, an environmental pollutant (including pesticides, insecticides, toxins, etc.); a chemical (including solvents, polymers, organic materials, etc.); therapeutic molecules (including therapeutic and abused drugs, antibiotics, etc.); biomolecules (including hormones, cytokines, proteins, nucleic acids, lipids, carbohydrates, cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, etc); whole cells (including procaryotic (such as pathogenic bacteria) and eukaryotic cells, including mammalian tumor cells); viruses (including retroviruses, herpesviruses, adenoviruses, lentiviruses, etc.); and spores; etc. Particularly preferred analytes are nucleic acids and proteins.

In a preferred embodiment, the target analyte is a protein. As will be appreciated by those in the art, there are a large number of possible proteinaceous target analytes that may be detected or evaluated for binding partners using the present invention. Suitable protein target analytes include, but are not limited to, (1) immunoglobulins; (2) enzymes (and other proteins); (3) hormones and cytokines (many of which serve as ligands for cellular receptors); and (4) other proteins.

In a preferred embodiment, the target analyte is a nucleic acid. These assays find use in a wide variety of applications. Such applications are described in more detail in USSNs 60/130,089, filed April 20, 1999; 60/160,927, filed October 22, 1999, 09/513,362, filed February 25, 2000, 60/135,051 filed May 20, 1999, 60/161,148, filed October 22, 1999, 09/517, 945, filed March 3, 2000, 09/425,633, filed October 22, 1999, 09/535,854, filed March 27, 2000, 09/535,993, filed April 20, 2000, 09/556,463, filed April 21, 2000, 60/224,119, filed October 26, 2000, all of which are expressly incorporated herein by reference.

In a preferred embodiment, the probes are used in genetic diagnosis. For example, probes can be made using the techniques disclosed herein to detect target sequences such as the gene for nonpolyposis colon cancer, the BRCA1 breast cancer gene, P53, which is a gene associated with a variety of cancers, the Apo E4 gene that indicates a greater risk of Alzheimer's disease, allowing for easy presymptomatic screening of patients, mutations in the cystic fibrosis gene, cytochrome p450s or any of the others well known in the art.

In an additional embodiment, viral and bacterial detection is done using the complexes of the invention. In this embodiment, probes are designed to detect target sequences from a variety of bacteria and viruses. For example, current blood-screening techniques rely on the detection of anti-HIV antibodies. The methods disclosed herein allow for direct screening of clinical samples to detect HIV nucleic acid sequences, particularly highly conserved HIV sequences. In addition, this allows direct monitoring of circulating virus within a patient as an improved method of assessing the efficacy of anti-viral therapies. Similarly, viruses associated with leukemia, HTLV-I and HTLV-II, may be detected in this way. Bacterial infections such as tuberculosis, chlamydia and other sexually transmitted diseases, may also be detected.

In a preferred embodiment, the nucleic acids of the invention find use as probes for toxic bacteria in the screening of water and food samples. For example, samples may be treated to lyse the bacteria to release its nucleic acid, and then probes designed to recognize bacterial strains, including, but not limited to, such pathogenic strains as, *Salmonella*, *Campylobacter*, *Vibrio cholerae*, *Leishmania*, enterotoxic strains of *E. coli*, and Legionnaire's disease bacteria. Similarly, bioremediation strategies may be evaluated using the compositions of the invention.

In a further embodiment, the probes are used for forensic "DNA fingerprinting" to match crime-scene DNA against samples taken from victims and suspects.

In an additional embodiment, the probes in an array are used for sequencing by hybridization.

The present invention also finds use as a methodology for the detection of mutations or mismatches in target nucleic acid sequences, as is described in more detail in USSNs 09/425,633, filed October 22, 1999, 09/535,854, filed March 27, 2000, both of which are expressly incorporated herein by reference. For example, recent focus has been on the analysis of the relationship between genetic variation and phenotype by making use of polymorphic DNA markers. Previous work utilized short tandem repeats (STRs) as polymorphic positional markers; however, recent focus is on the use of single nucleotide polymorphisms (SNPs). Common SNPs occur at an average frequency of more than 1 per kilobase in human genomic DNA. Some SNPs, particularly those in and around coding sequences, are likely to be the direct cause of therapeutically relevant phenotypic variants. There are a number of well known polymorphisms that cause clinically important phenotypes; for example, the apoE2/3/4 variants are associated with different relative risk of Alzheimer's and other diseases (see Cordor et al., Science

261(1993). Multiplex PCR amplification of SNP loci with subsequent hybridization to oligonucleotide arrays has been shown to be an accurate and reliable method of simultaneously genotyping at least hundreds of SNPs; see Wang et al., Science, 280:1077 (1998); see also Schafer et al., Nature Biotechnology 16:33-39 (1998). The compositions of the present invention may easily be substituted for the arrays of the prior art.

In a preferred embodiment, the compositions of the invention are used to screen bioactive agents to find an agent that will bind, and preferably modify the function of, a target molecule. As above, a wide variety of different assay formats may be run, as will be appreciated by those in the art. Generally, the target analyte for which a binding partner is desired is labeled; binding of the target analyte by the bioactive agent results in the recruitment of the label to the bead, with subsequent detection.

In a preferred embodiment, the binding of the bioactive agent and the target analyte is specific; that is, the bioactive agent specifically binds to the target analyte. By "specifically bind" herein is meant that the agent binds the analyte, with specificity sufficient to differentiate between the analyte and other components or contaminants of the test sample. However, as will be appreciated by those in the art, it will be possible to detect analytes using binding which is not highly specific; for example, the systems may use different binding ligands, for example an array of different ligands, and detection of any particular analyte is via its "signature" of binding to a panel of binding ligands, similar to the manner in which "electronic noses" work. This finds particular utility in the detection of chemical analytes. The binding should be sufficient to remain bound under the conditions of the assay, including wash steps to remove non-specific binding, although in some embodiments, wash steps are not desired; i.e. for detecting low affinity binding partners. In some embodiments, for example in the detection of certain biomolecules, the dissociation constants of the analyte to the binding ligand will be less than about 10^{-4}-10^{-6} M^{-1}, with less than about 10^{-5} to 10^{-9} M^{-1} being preferred and less than about 10^{-7} -10^{-9} M^{-1} being particularly preferred.

Generally, a sample containing a target analyte (whether for detection of the target analyte or screening for binding partners of the target analyte) is added to the array, under conditions suitable for binding of the target analyte to at least one of the bioactive agents, i.e. generally physiological conditions. The presence or absence of the target analyte is then detected. As will be appreciated by those in the art, this may be done in a variety of ways, generally through the use of a change in an optical signal. This change can occur via many different mechanisms. A few examples include the binding of a dye-tagged analyte to the bead, the production of a dye species on or near the beads, the destruction of an existing dye species, a change in the optical signature upon analyte interaction with dye on bead, or any other optically interrogatable event.

In a preferred embodiment, the change in optical signal occurs as a result of the binding of a target analyte that is labeled, either directly or indirectly, with a detectable label, preferably an optical label such as a fluorochrome. Thus, for example, when a proteinaceous target analyte is used, it may be

either directly labeled with a fluor, or indirectly, for example through the use of a labeled antibody. Similarly, nucleic acids are easily labeled with fluorochromes, for example during PCR amplification as is known in the art. Alternatively, upon binding of the target sequences, a hybridization indicator may be used as the label. Hybridization indicators preferentially associate with double stranded nucleic acid, usually reversibly. Hybridization indicators include intercalators and minor and/or major groove binding moieties. In a preferred embodiment, intercalators may be used; since intercalation generally only occurs in the presence of double stranded nucleic acid, only in the presence of target hybridization will the label light up. Thus, upon binding of the target analyte to a bioactive agent, there is a new optical signal generated at that site, which then may be detected.

Alternatively, in some cases, as discussed above, the target analyte such as an enzyme generates a species that is either directly or indirectly optically detectable.

Furthermore, in some embodiments, a change in the optical signature may be the basis of the optical signal. For example, the interaction of some chemical target analytes with some fluorescent dyes on the beads may alter the optical signature, thus generating a different optical signal.

As will be appreciated by those in the art, in some embodiments, the presence or absence of the target analyte may be done using changes in other optical or non-optical signals, including, but not limited to, surface enhanced Raman spectroscopy, surface plasmon resonance, radioactivity, etc.

The assays may be run under a variety of experimental conditions, as will be appreciated by those in the art. A variety of other reagents may be included in the screening assays. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used. The mixture of components may be added in any order that provides for the requisite binding. Various blocking and washing steps may be utilized as is known in the art.

In a preferred embodiment, two-color competitive hybridization assays are run. These assays can be based on traditional sandwich assays. The beads contain a capture sequence located on one side (upstream or downstream) of the SNP, to capture the target sequence. Two SNP allele-specific probes, each labeled with a different fluorophor, are hybridized to the target sequence. The genotype can be obtained from a ratio of the two signals, with the correct sequence generally exhibiting better binding. This has an advantage in that the target sequence itself need not be labeled. In addition, since the probes are competing, this means that the conditions for binding need not be optimized. Under conditions where a mismatched probe would be stably bound, a matched probe can still displace it. Therefore the competitive assay can provide better discrimination under those conditions. Because many assays are carried out in parallel, conditions cannot be optimzed for every probe

simultaneously. Therefore, a competitive assay system can be used to help compensate for non-optimal conditions for mismatch discrimination.

In a preferred embodiment, dideoxynucleotide chain-termination sequencing is done using the compositions of the invention. In this embodiment, a DNA polymerase is used to extend a primer using fluorescently labeled ddNTPs. The 3' end of the primer is located adjacent to the SNP site. In this way, the single base extension is complementary to the sequence at the SNP site. By using four different fluorophors, one for each base, the sequence of the SNP can be deduced by comparing the four base-specific signals. This may be done in several ways. In a first embodiment, the capture probe can be extended; in this approach, the probe must either be synthesized 5'-3' on the bead, or attached at the 5' end, to provide a free 3' end for polymerase extension. Alternatively, a sandwich type assay can be used; in this embodiment, the target is captured on the bead by a probe, then a primer is annealed and extended. Again, in the latter case, the target sequence need not be labeled. In addition, since sandwich assays require two specific interactions, this provides increased specificity which is particularly helpful for the analysis of complex samples.

In addition, when the target analyte and the DBL both bind to the agent, it is also possible to do detection of non-labelled target analytes via competition of decoding.

In a preferred embodiment, the methods of the invention are useful in array quality control. Prior to this invention, no methods have been described that provide a positive test of the performance of every probe on every array. Decoding of the array not only provides this test, it also does so by making use of the data generated during the decoding process itself. Therefore, no additional experimental work is required. The invention requires only a set of data analysis algorithms that can be encoded in software.

The quality control procedure can identify a wide variety of systematic and random problems in an array. For example, random specks of dust or other contaminants might cause some sensors to give an incorrect signal-this can be detected during decoding. The omission of one or more agents from multiple arrays can also be detected; that is, since the arrays formulated herein are randomly self-assembled, arrays comprising different statistical distributions of each sensor elements may be made; the invention allows actual QC on the arrays, for "sorting" arrays with good distributions from those with less than ideal distributions. These procedures also allow the determination of sites that do not comprise beads. This quality control procedure can also be used in non-random arrays.

An advantage of this quality control procedure is that it can be implemented immediately prior to the assay itself (or after, in some instances), and is a true functional test of each individual sensor. Therefore any problems that might occur between array assembly and actual use can be detected. In applications where a very high level of confidence is required, and/or there is a significant chance of

sensor failure during the experimental procedure, decoding and quality control can be conducted both before and after the actual sample analysis.

In an alternative method of quality control, a single fiber bundle of an array is dedicated to quality control. That is a dedicated bundle or subset of fibers within an array is independently analyzed for quality control. In one embodiment, the control fiber(s) are targeted or labeled with a unique code or label. At each state of hybridization, in addition to the experimental probes, the quality control probes are hybridized with the array. Upon taking the image from the first hybridization of the first stage, the intensity of the control fiber(s) is analyzed. An aberrant reading or signal from the control fiber indicates a failed or at least non-optimal hybridization step.

In a preferred embodiment, the arrays can be used to do reagent quality control. In many instances, biological macromolecules are used as reagents and must be quality controlled. For example, large sets of oligonucleotide probes may be provided as reagents. It is typically difficult to perform quality control on large numbers of different biological macromolecules. For example, when large populations of different random oligonucleotides are synthesized, it may be desirable to check that each population is represented. The approach described here can be used to do this by treating the reagents (formulated as the DBLs) as variable instead of the arrays.

In a preferred embodiment, the methods outlined herein are used in array calibration. For many applications, such as mRNA quantitation, it is desirable to have a signal that is a linear response to the concentration of the target analyte, or, alternatively, if non-linear, to determine a relationship between concentration and signal, so that the concentration of the target analyte can be estimated. For example, different probes can show differences in hybridization efficiencies, etc., and it may be desirable to choose appropriate probes under experimental conditions. Thus, for example, when expression profiling is done, and quantitation is desirable, calibration curves can be done to see how individual probes react, and then the probes that give the best response (i.e. linearity at the concentrations and conditions of interest) can be chosen for further assays. Accordingly, the present invention provides methods of creating calibration curves in parallel for multiple beads in an array. The calibration curves can be created under conditions that simulate the complexity of the sample to be analyzed. Each curve can be constructed independently of the others (e.g. for a different range of concentrations), but at the same time as all the other curves for the array.

Thus, in these embodiments, different types of experiments can be done. For example, the sequential decoding scheme can be implemented with different concentrations being used as the code "labels", rather than different fluorophores. In this way, signal as a response to concentration can be measured for each bead. This calibration can be carried out just prior to array use, so that every probe on every array is individually calibrated as needed. Alternatively, different concentrations of the decoding probe can have different labels.

It should be noted that the assay calibration methods find use in non-random arrays, as well; that is, other types of support-bound nucleic acid arrays can be calibrated using these methods as well. Thus, for example, the sequential addition of different pools of probes, wherein the concentration of the probes is varied, to biochips can allow calibration of any assay system. This type of analysis can also be done on non-random arrays for quality control, to verify the integrity and sequence of the support bound probes, and in assay development to identify good probes.

In a preferred embodiment, the methods of the invention can be used in assay development as well. Thus, for example, the methods allow the identification of good and bad probes; as is understood by those in the art, some probes do not function well because they do not hybridize well, or because they cross-hybridize with more than one sequence. These problems are easily detected during decoding. The ability to rapidly assess probe performance has the potential to greatly reduce the time and expense of assay development. Thus, probes that respond linearly with concentration, show low non-specific binding, or give signals in a particular range, can be chosen for addition to a new array for assays.

Similarly, in a preferred embodiment, the methods of the invention are useful in quantitation in assay development. Major challenges of many assays is the ability to detect differences in analyte concentrations between samples, the ability to quantitate these differences, and to measure absolute concentrations of analytes, all in the presence of a complex mixture of related analytes. An example of this problem is the quantitation of a specific mRNA in the presence of total cellular mRNA. One approach that has been developed as a basis of mRNA quantitation makes use of a multiple match and mismatch probe pairs (Lockhart et al., 1996), hereby incorporated by reference in its entirety. While this approach is simple, it requires relatively large numbers of probes. In this approach, a quantitative response to concentration is obtained by averaging the signals from a set of different probes to the gene or sequence of interest. This is necessary because only some probes respond quantitatively, and it is not possible to predict these probes with certainty. In the absence of prior knowledge, only the average response of an appropriately chosen collection of probes is quantitative. However, in the present invention, that can be applied generally to nucleic acid based assays as well as other assays. In essence, the approach is to identify the probes that respond quantitatively in a particular assay, rather than average them with other probes. This is done using the array calibration scheme outlined above, in which concentration-based codes are used. Advantages of this approach include: fewer probes are needed; the accuracy of the measurement is less dependent on the number of probes used; and that the response of the sensors is known with a high level of certainty, since each and every sequence can be tested in an efficient manner. It is important to note that probes that perform well are selected empirically, which avoids the difficulties and uncertainties of predicting probe performance, particularly in complex sequence mixtures. In contrast, in experiments described to date with ordered arrays, relatively small numbers of sequences are checked by performing quantitative spiking experiments, in which a known mRNA is added to a mixture.

In a preferred embodiment, cDNA arrays are made for RNA expression profiling. In this embodiment, individual cDNA clones are amplified (for example, using PCR) from cDNA libraries propagated in a host-vector system. Each amplified DNA is attached to a population of beads. Different populations are mixed together, to create a collection of beads representing the cDNA library. The beads are arrayed, decoded as outlined above, and used in an assay (although as outlined herein, decoding may occur after assay as well). The assay is done using RNA samples (whole cell or mRNA) that are extracted, labeled if necessary, and hybridized to the array. Comparative analysis allows the detection of differences in the expression levels of individual RNAs. Comparison to an appropriate set of calibration standards allows quantification of absolute amounts of RNA.

The cDNA array can also be used for mapping, e.g. to map deletions/insertions or copy number changes in the genome, for example from tumors or other tissue samples. This can be done by hybridizing genomic DNA. Instead of cDNAs (or ESTs, etc.), other STS (sequence tagged sites), including random genomic fragments, can also be arrayed for this purpose.

In one embodiment, the invention provides a method of single nucleotide polymorphism (SNP) genotyping. As described herein and in U.S.S.N.s 09/553,993 filed April 20, 2000, 09/556,463 filed April 21, 2000 and 60/244,119, filed October 26, 2000, all of which are expressly incorporated herein by reference, a target nucleic acid is contacted with a probe that is specific for a particular allele of a single nucleotide polymorphism. The target nucleic acid can be immobilized prior to contacting with the probe, or alternatively, the probe: target hybridization can occur in solution following by immobilization of the probe: target nucleic acid complex. Alternatively, a capture prove on a bead may be specific for a particular SNP. What is important in this invention is the nature of the probe for detecting a SNP.

There are four possibilities for the bases at a polymorphic site: -A, T, G, or C. However, it is known that most of the human SNP's are dimorphic, i.e., they only include the combinations of two of the four bases. As such, the prior art generally relied on distinguishing between two alleles that are present within a SNP. and characterized them as either "AA", "AB, or "BB".

However, according to the present invention, the probes are designed such that each of the four possible nucleotides that could be present at a particular position are included in the SNP probes. In addition, the probes are labeled with a specific color that corresponds with the nucleotide that hybridizes to the nucleotide to be interrogated. For instance, all the alleles that correspond to an A-polymorphic site are labeled with red. Similarly, all the alleles that correspond to a G-polymorphic site are labeled green, etc. As such the true identity of the polymorphism is determined. That is, if the polymorphism is of the type GT, one would not classify is as "AB" genotype. Instead, the GT code will come up directly from the genotyping routine.

An advantage of this method over the prior art is the capability of independent error checking. That is, knowing that polymorphisms are typically dimorphic, by using probes that correspond to all possibilities of polymorphisms at a sites, one obtains a check against false results, for example of a signal is detected that does not correspond with the known polymorphisms. That is, in the prior art, the resultant genotyping would be one of the following three possibilities: AA, AB, or BB. However, by using this invention, the number of possible results is increased from 3 to 10: AA, AG, AC, AT, GG, GC, GT, CC, CT, TT . Of these ten possibilities, only 3 are the possible combinations for each SNP, e.g., a T/G polymorphism can only render one of the following three possibilities: TT, TG, or GG. Therefore, in this case, if the computed genotype falls into any of the remaining 10-3=7 combinations (e.g., either of AA, AG, AC, AT, GC, CC, CT), it would be a clear indication that an error has occurred.

All references cited herein are incorporated by reference in their entirety.

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are incorporated by reference in their entirety.

Examples

Example 1

Sixteen microspheres (beads) were labeled combinatorially with two different fluorophores (FAM and Cy3). In a first round of labeling, either FAM or Cy3 labeled oligonucleotides that were complementary to the oligonucleotide (IBL) on the microsphere, were hybridized with the microsphere. Labeling of oligonucleotides was performed as is well known in the art. Hybridization conditions are known in the art.

Following a first round of hybridization, the two pools of beads were divided into two pools each and each labeled either with the FAM or Cy3 labeled oligonucleotide. This process was repeated two additional times. Thus, following four successive rounds of labeling, each microsphere was labeled with a unique code (see Figure 1). The identity of each microsphere was elucidated by determining the identity of each fluorophore in succession; the terminal fluorophere was determined and then removed to allow for the identification of the next fluorophore. In this fashion, with as few as 4 decoding steps, the identity of 16 microspheres is determined.

Example 2

A decoding scheme similar to that described in Example 1 was implemented for four color decoding. In this example, beads were labeled as described in Example 1 with the exception that 4 labels were used at each stage. 4013 beads were labeled using Bod493, BodR6G, Bod564 and BodTXR labeled oligonucleotides. 128 different bead types were identified based on the successive decoding of the four colors.

Example 3

An alternative method to using multiple colors is to use ratiometric intensities as a coding scheme. A normalizing image is acquired in which every bead exhibits its "full" intensity. Subsequent decode stages generate intensity codes by hybridizing mixtures of "labeled":"unlabeled" complementary oligonucleotides. For instance, Fig. 1 depicts three different intensity shades (low, medium, and high) which can be ratioed to a stage with all complements present at a "high" shading value. An experiment using grey scale decoding on 16 different bead types is shown in Fig. 3.

Figure 3A depicts the combinatorial pooling scheme for labeling beads with different ratios of labeled oligonucleotides. A particular oligo is present at either 100% Cy3-labeled, 40% Cy3-labeled (60% unlabeled), or 10% Cy3-labeled (90% unlabeled) fraction. Decode oligos were hybridized to the array

for 2 min. at a 50 nM concentration. Subsequently, two independent normalizing images (all oligo complements are present as 100% Cy3-labeled species) were acquired, and the resulting bead intensities compared. This is depicted in Figure 3B as the normalized values are plotted against each other. Finally, to identify or decode the beads, the alpha values (ratio of bead intensity in indicated decode stage to intensity in normalization image) are plotted for three decode stages described in (A). In stage 1, only two peaks are observed in the alpha value histogram since only 16 bead types are present on the array. Three distinguishable peaks are observed in the second and third decode stages indicating the feasibility of grey scale decoding.

Physical attributes and different "levels" of the attributes can be used as codes by which to distinguish bead types from another. Thus, for an attribute to act as a robust code, it should be possible to imbue a bead with different "levels" of a particular attribute. Each "level" of an attribute should be quantitatively well separated from other "levels". The important point is to maximize the dynamic range of the attribute measurement, and minimize the spread of the measurement.

Example 4

For a given oligo sequence, several codes can be generated by varying the distance of separation of the two fluorophores . As shown in Figure 4, a complementary sequence containing FRET dyes separated by a linker of varying lengths allows multiple codes to be generated (Figure 4A). As an example, Probe A and B are hybridized to a complementary sequence immobilized on a solid support. Probe A contains dyes separated by an 8 poly T linker, whereas probe B is hybridized to a linker with a 4 poly T linker. The efficiency of energy transfer (ratio of intensities of dye2:dye1) is greater for probe B than for probe A (Figure 4B).

Example 5

The following FRET oligonucleotides (probes ET1, ET2, ET5 and ET7) were synthesized as is known in the art and labeled with Cy3 and fluorscein separated by linkers of varying length:

ET1 T**G*CACGAGAATGGAGGTATCT
ET2 C**TGTCGC*ACGAGAATGGAGGTATCT
ET5 C**TGTCGGGGCACTCATTTGTGC*ACGAGAATGGAGGTATCT
ET7 C**TGTCGGGGCACTCATTTGTCTGTCGGGGCGC*ACGAGAATGGAGGTATCT.

**is Cy3 * is fluorescein

An aliquot of each FRET oligonucleotide was assayed as is known in the art. Emission spectra were normalized relative to emission at 530nm. As can be seen in Figure 5 emission of fluorescein increases as the linker is shortened. The ratio of intensities of the two fluorophores, therefore, is characteristic of the distance between the fluorophores.

Example 6

To illustrate the primer extension decoding, a two-color model is illustrated in Figure 9. To decode 16 different bead addresses using only two colors. 16 different beads are labeled by attaching 16 different unique oligos (IBLs) to each of 16 batches of beads and pooling the bead batches together. Along with the beads 16 oligos (DBLs) that are complementary to these IBLs are synthesized. Each unique IBL used in this method contains two domains. The domains are composed of a binding domain and an extension domain. The pooled bead populations are then self-assembled onto a fiber optic tip end as described herein. After array self-assembly a combinatorial color decoding process is initiated as follows: A primer extension reaction is initiated at the end of the fiber bundle tip containing the arrayed beads by simultaneously hybridizing 16 complementary oligos (DBLs) to their cognate IBL. In the first extension stage, the DBLs would have exactly the same length (n) as the binding domain of the IBL . The first stage primer extension reaction would contain 16 primer oligos, a DNA polymerase, appropriate buffer, and two fluorescently-labeled dideoxynucleotide terminators. During the extension reaction, hybridized primers are extended by a single nucleotide complementary to the first nucleotide in the extension domain of the immobilized IBL. The described extension reaction 'paints' or labels any given bead with one of two possible fluorescent terminator colors. After the reaction is over the array tip is imaged to capture the color of the beads at this stage. Beads are then immediately stripped of color or label by denaturing and washing off the first extended primer at each zip code loci. This process can be repeated through multiple stages. In subsequent stages, the primer extension reactions contain the same reagents as the previous extension reaction except that the binding complementary oligos are one base longer. These (n+1) primers hybridize with their cognate binding domain and one base into the 'extension domain'. Figure 9 illustrates a four-stage extension process (2-color) whereby each stage allows for the assignment of a unique combinatorial color-coded 'address' to each bead type.

In addition, if this primer-based extension method were to be combined with degenerate DBL-BL combinations for decoding large combinatorial libraries, for example using the CVC (constant-variable-constant domain idea), as described herein, the extension reaction would extend into one of 10 different possible nucleotide combinations in a ' variable extension region'. This would require only three extension reactions to decodify 1000 unique addresses (i.e. A = lot where L is the length of the variable region). The combination of the degenerate DBL-IBL combinations such as CVC with the primer extension method is illustrated in Figure 10.

Example 7

This method relates to fluorescent based encoding and decoding process based on the fact that fluorescent dyes have broad band absorption and emission spectra. Fewer detection channels are used than the number of encoding dyes used in the process. That is, for n detection channels, a particular dye, x, can be represented by a vector I_x in the n -dimensional space.

$$I_x \{ \frac{I}{\int_i^n I_i} \perp I_1 I_2 I_3 .... I_n$$

where I_i is the intensity detected at channel i. Theoretically, an infinite number of vectors are possible in the n-dimensional space when n is larger or equal to 2. With an appropriate dye selection and optical filter design, for any given number of detection channels, a larger number of dyes can be used for decoding. For example, to distinguish three species, it is possible to tag the species with three different dyes, x, y, z and use only two detection channels, such that

$I_x = \{1 \quad 0\}$
$I_y = \{a \quad 1-a\} \quad 0< a <1$
$I_z = \{ 0 \quad 1\}$

Figure 11 illustrates the experimental results for this example scheme used in decoding. Three classes of beads were tagged with Bodipy-493, Bodipy-R6G and Bodipy564. Detection channel 1 consisted of a 410nm/20 excitation filter and a 540nm/20 emission filter. Detection channel 2 consisted of a 535nm/20 excitation filter and a 585nm/nm emission filter. Excited with a xenon arc lamp, the fluorescence emission of Bodipy-493 can only be detected in channel 1. Bodipy-564 emission can be detected in channel 2. Bodipy-R6G can be detected in both channels. As expected, the scatter plot (Figure 11) of the intensity in the two channels of the beads clearly indicates three classes. Within each class, the beads have the same relative intensity ratio (corresponding to the same unit vector) with a certain uncertainties.

Similar design and data analysis procedure can be used for other dyes and detection channels.

CLAIMS

We claim:

1. A method of decoding an array composition comprising

a) providing an array composition comprising a population of microspheres comprising at least 500 subpopulations, wherein each subpopulation comprises a bioactive agent;

b) adding a plurality of decoding binding ligands to said population of microspheres to identify at least 500 of the bioactive agents.

2. A method according to claim 1 wherein at least one subpopulation of microspheres comprises an identifier binding ligand to which a decoding binding ligand can bind.

3. A method according to claim 1 or 2 wherein said decoding binding ligands bind to said bioactive agents.

4. A method according to claim 1, 2 or 3, wherein said decoding binding ligands are labeled.

5. A method according to claim 1, 2, 3 or 4, wherein the is bioactive agent of each subpopulation is determined.

6. A method according to claim 1,2, 3, 4 or 5, wherein said array composition further comprises a substrate with a surface comprising discrete sites, wherein said microspheres are randomly distributed on said substrate.

7. A method according to claim 1,2, 3, 4 or 5, wherein said array composition is a liquid array.

8. A method according to claim 1,2, 3, 4 or 5, wherein each subpopulation comprises a bioactive agent and does not comprise an optical signature

9. A method according to claim 1, 2, 3, 4, 5, 6, 7 or 8. wherein said bioactive agents are nucleic acids.

10. A method according to claim 1, 2, 3, 4, 5, 6, 7 or 8 wherein said bioactive agents are proteins.

11. A method according to claim 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, wherein said population comprises at least 1000 subpopulations.

12. A method according to claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11, wherein said population comprises at least 1500 subpopulations.

13. A method of determining the presence of a target analyte in a sample comprising:
a) contacting said sample with a composition comprising a population of microspheres comprising at least 500 subpopulations, wherein each subpopulation comprises a bioactive agent;
b) adding a plurality of decoding binding ligands to said population of microspheres to identify at least 500 of the bioactive agents; and
c) determining the presence or absence of said target analyte.

14. A method according to claim 13, wherein said subpopulations are distributed on a substrate.

15. A method according to claim 14 wherein said array is a liquid array.

16. A method according to claim 15, wherein said determining is by FACS.

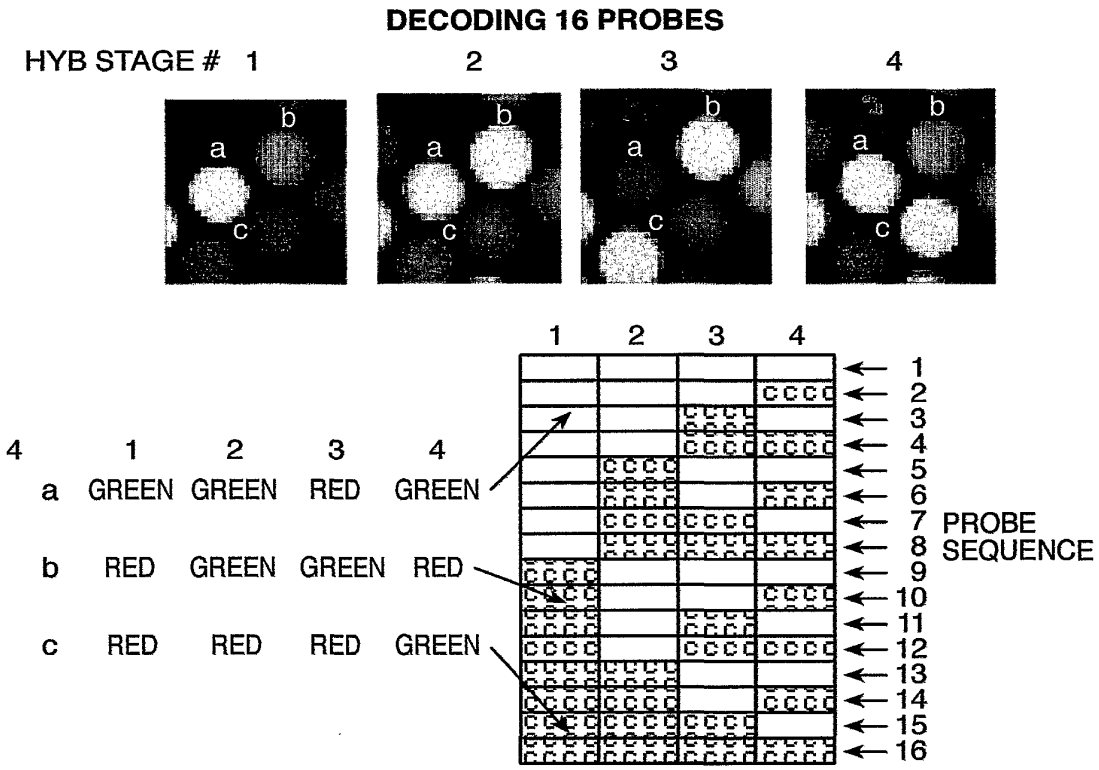


FIG._1

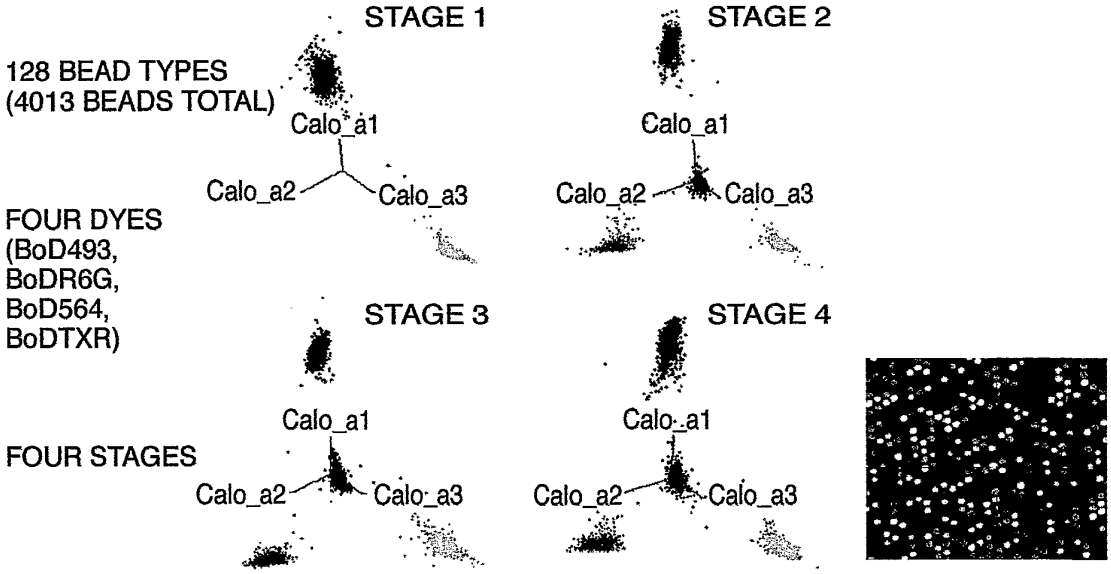


FIG._2

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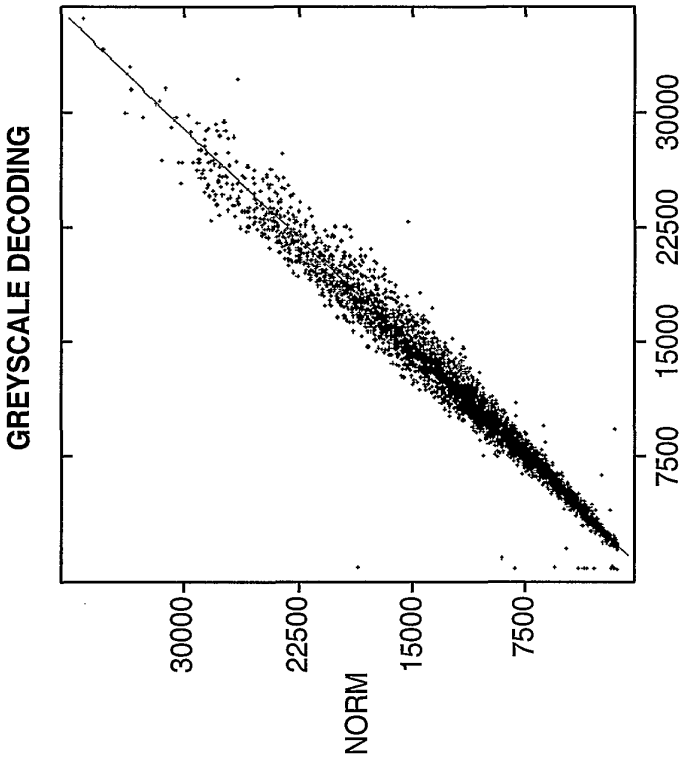


FIG._3B

GREYSCALE DECODING

CODE	S1	S2	S3
1	100	100	100
2	100	100	40
3	100	100	10
4	100	40	100
5	100	40	40
6	100	40	10
7	100	10	100
8	100	10	40
9	100	10	10
10	40	100	100
11	40	100	40
12	40	100	10
13	40	40	100
14	40	40	40
15	40	40	10
16	40	10	100
17	40	10	40
18	40	10	10
19	10	100	100
20	10	100	40
21	10	100	10
22	10	40	100
23	10	40	40
24	10	40	10
25	10	10	100
26	10	10	40
27	10	10	10

FIG._3A

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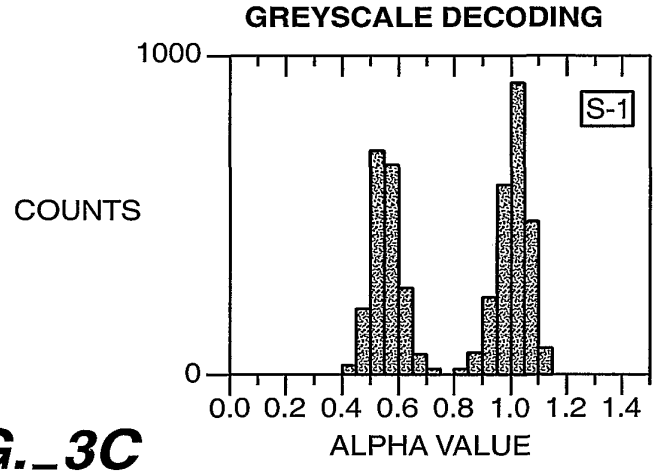


FIG._3C

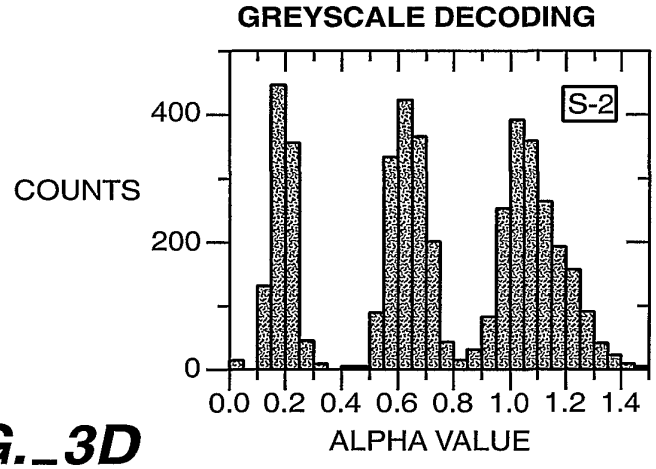


FIG._3D

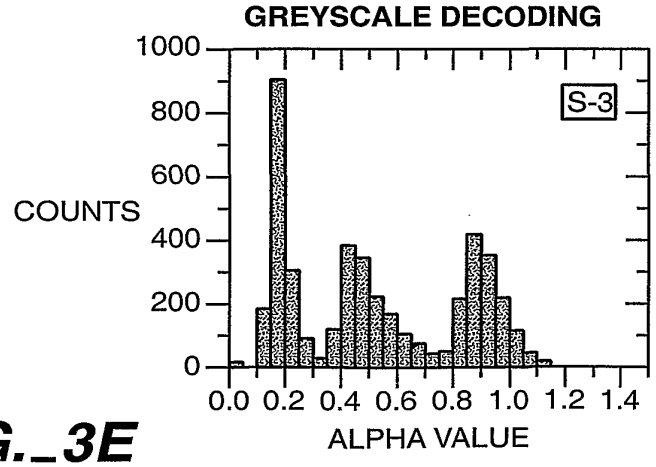


FIG._3E

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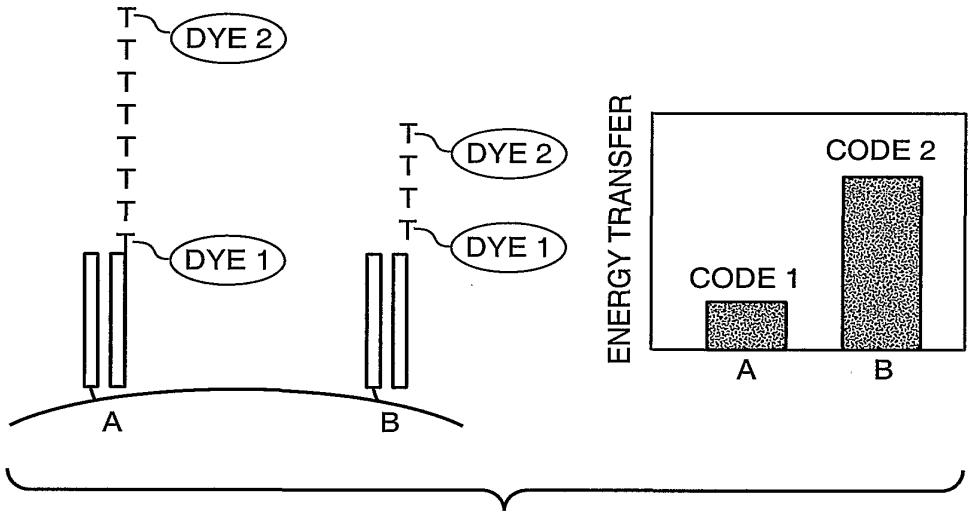


FIG._4

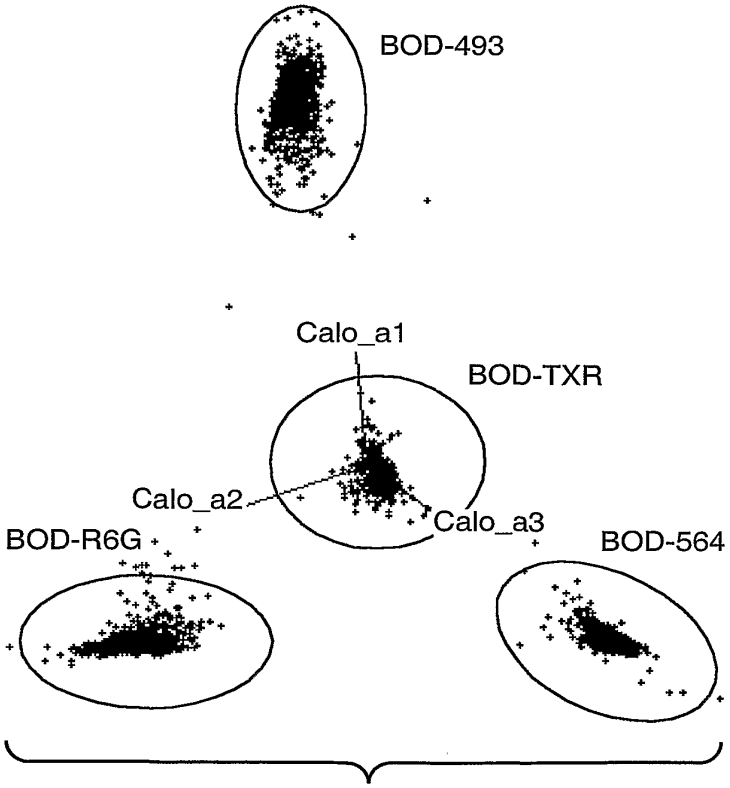


FIG._6

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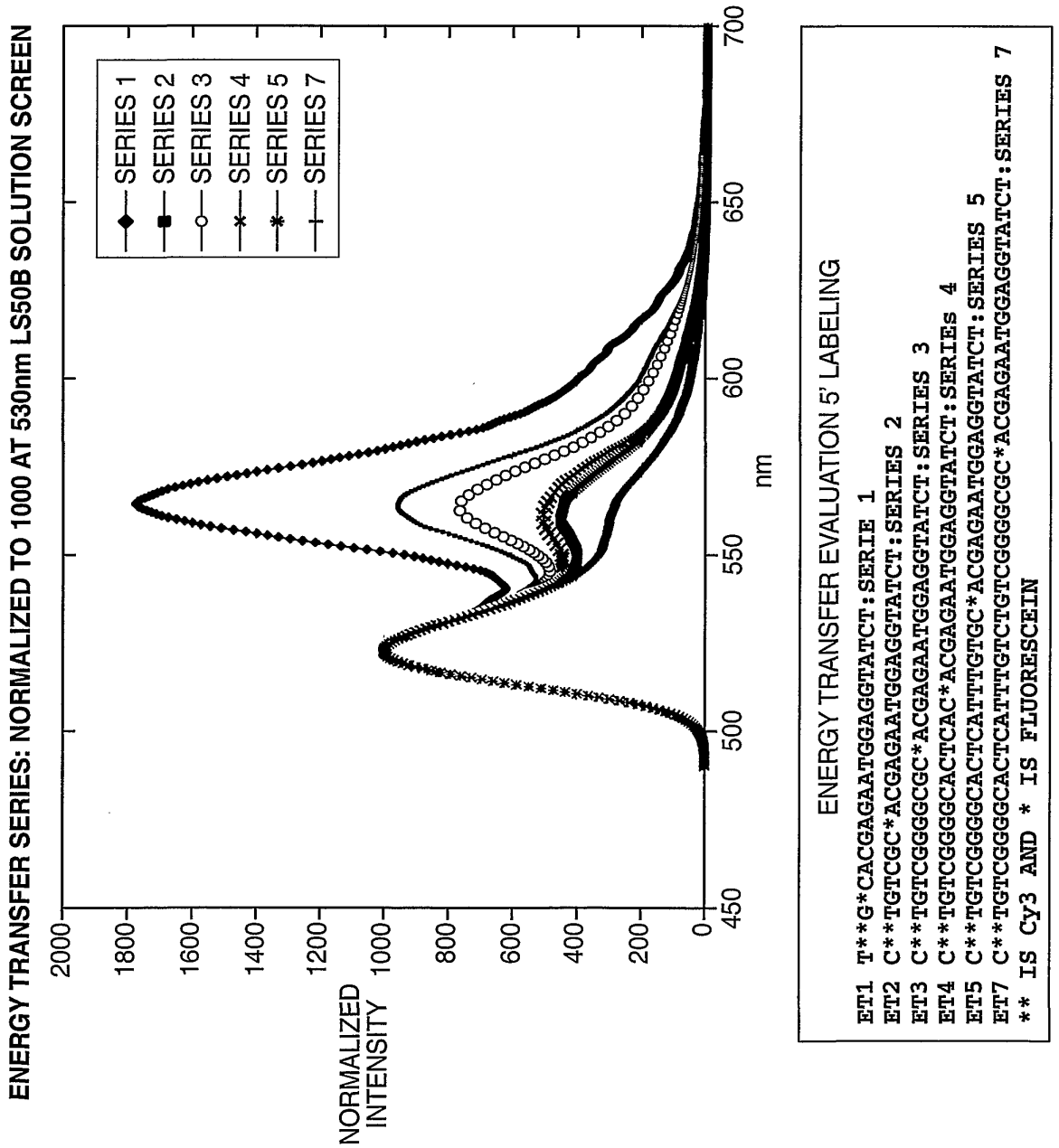


FIG._5

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	1 "COLOR" DECODING					
BEAD	STAGE 1	STAGE 2	STAGE 3	STAGE 4	CODE	PARITY
1	1	1	1	1	1111	EVEN
	1	1	1		1110	ODD
	1	1		1	1101	ODD
2	1	1			1100	EVEN
	1		1	1	1011	ODD
3	1		1		1010	EVEN
4	1			1	1001	EVEN
	1				1000	ODD
		1	1	1	0111	ODD
5		1	1		0110	EVEN
6		1		1	0101	EVEN
		1			0100	ODD
7			1	1	0011	EVEN
			1		0010	ODD
				1	0001	ODD
8					0000	EVEN

1001 DIGIT SUM = 2, EVEN PARITY

↓

1011 DIGIT SUM = 3, ODD PARITY

FIG._7

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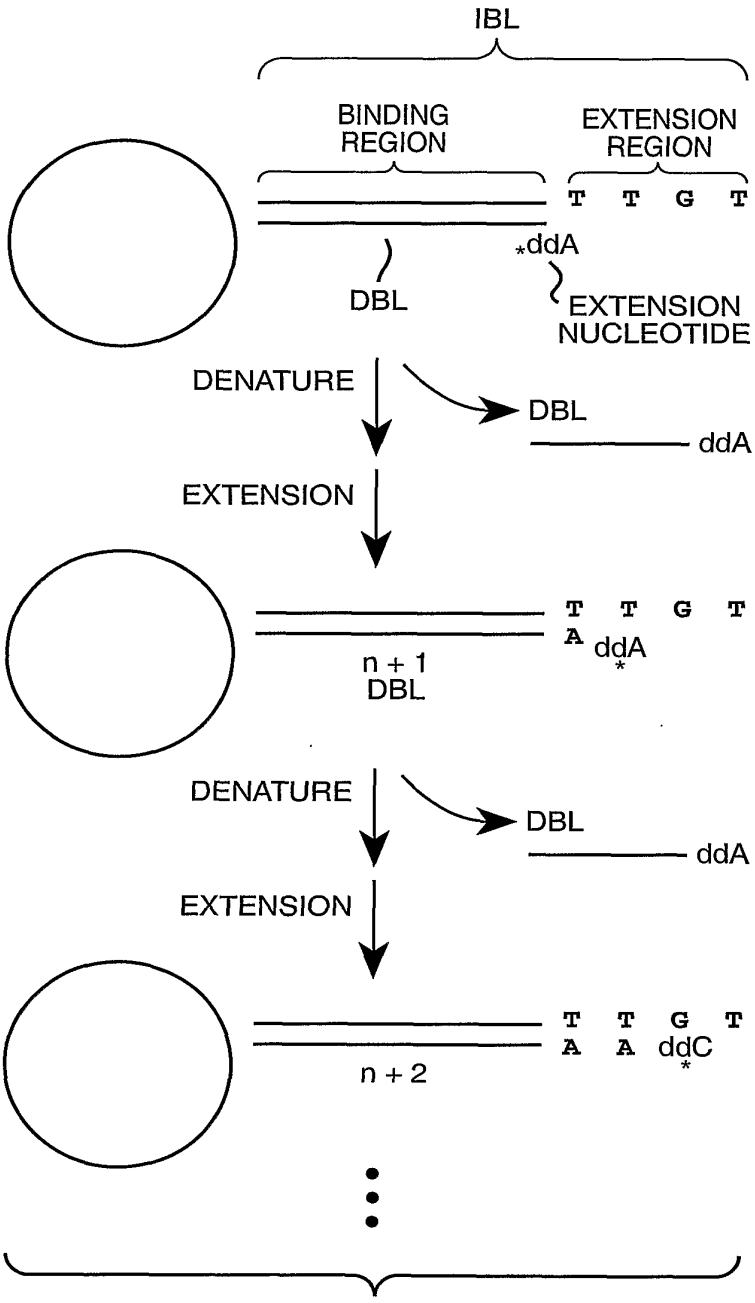


FIG._8

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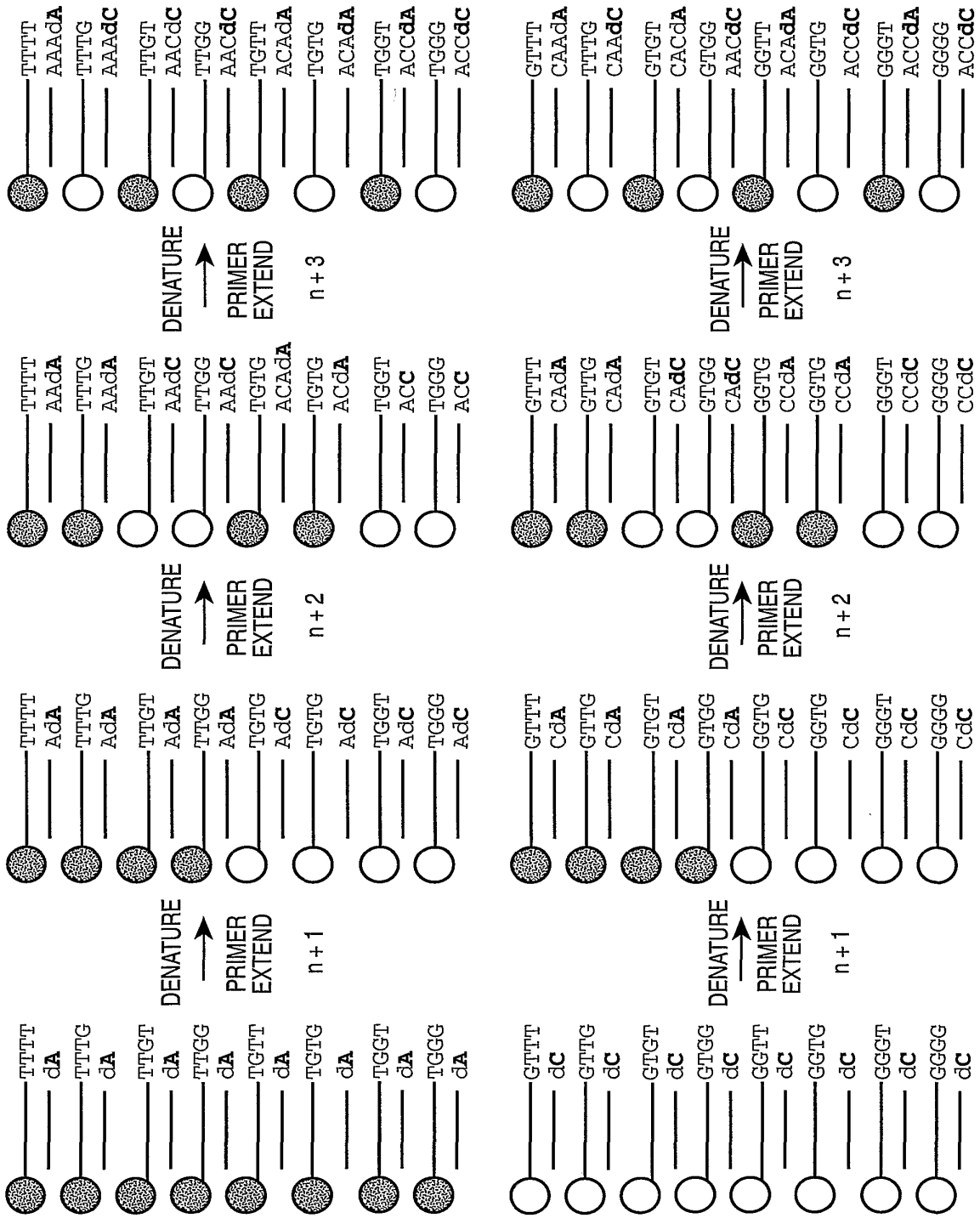


FIG._9

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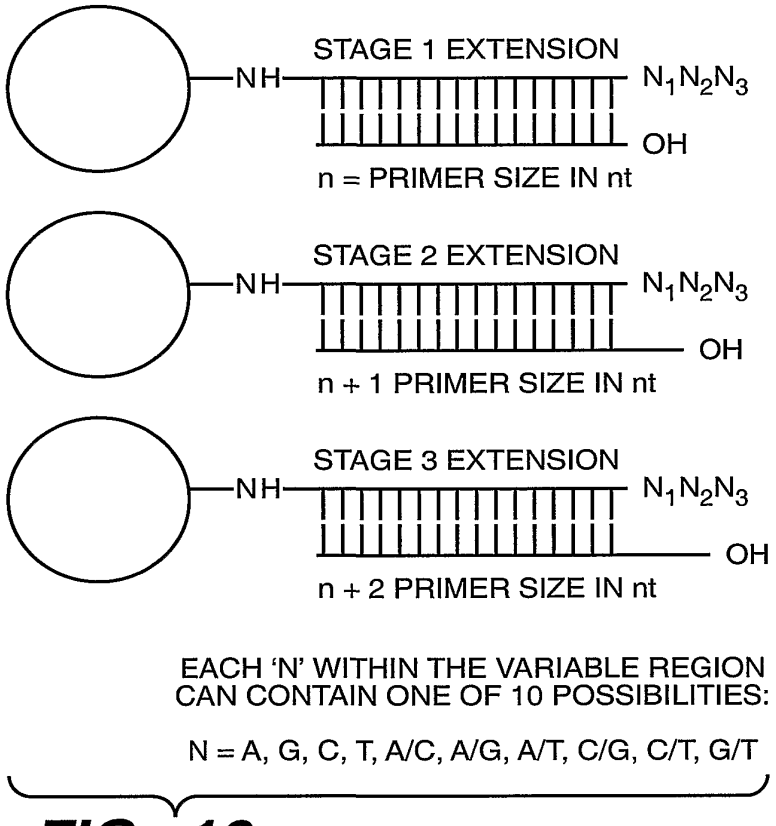


FIG._10

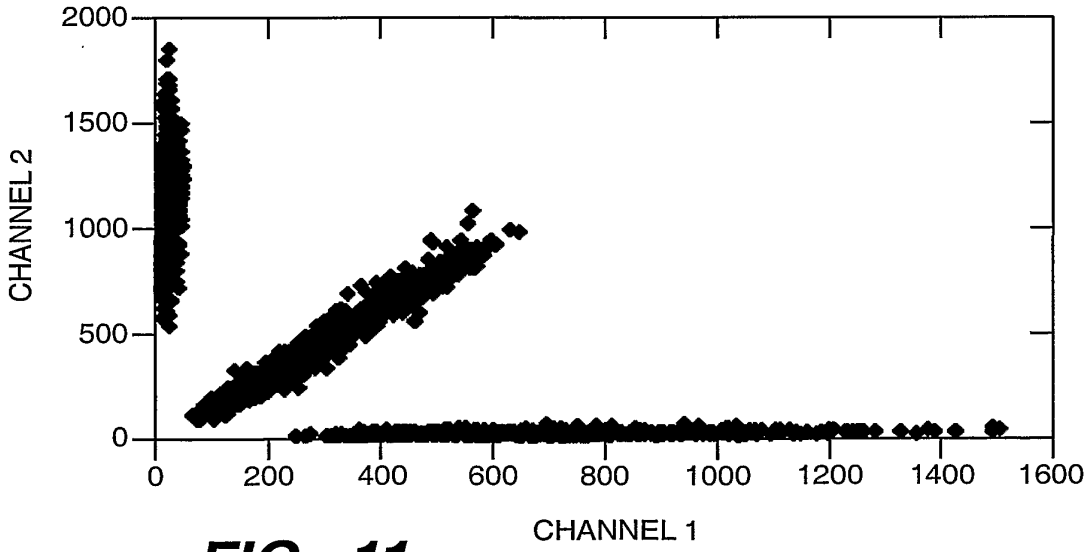


FIG._11

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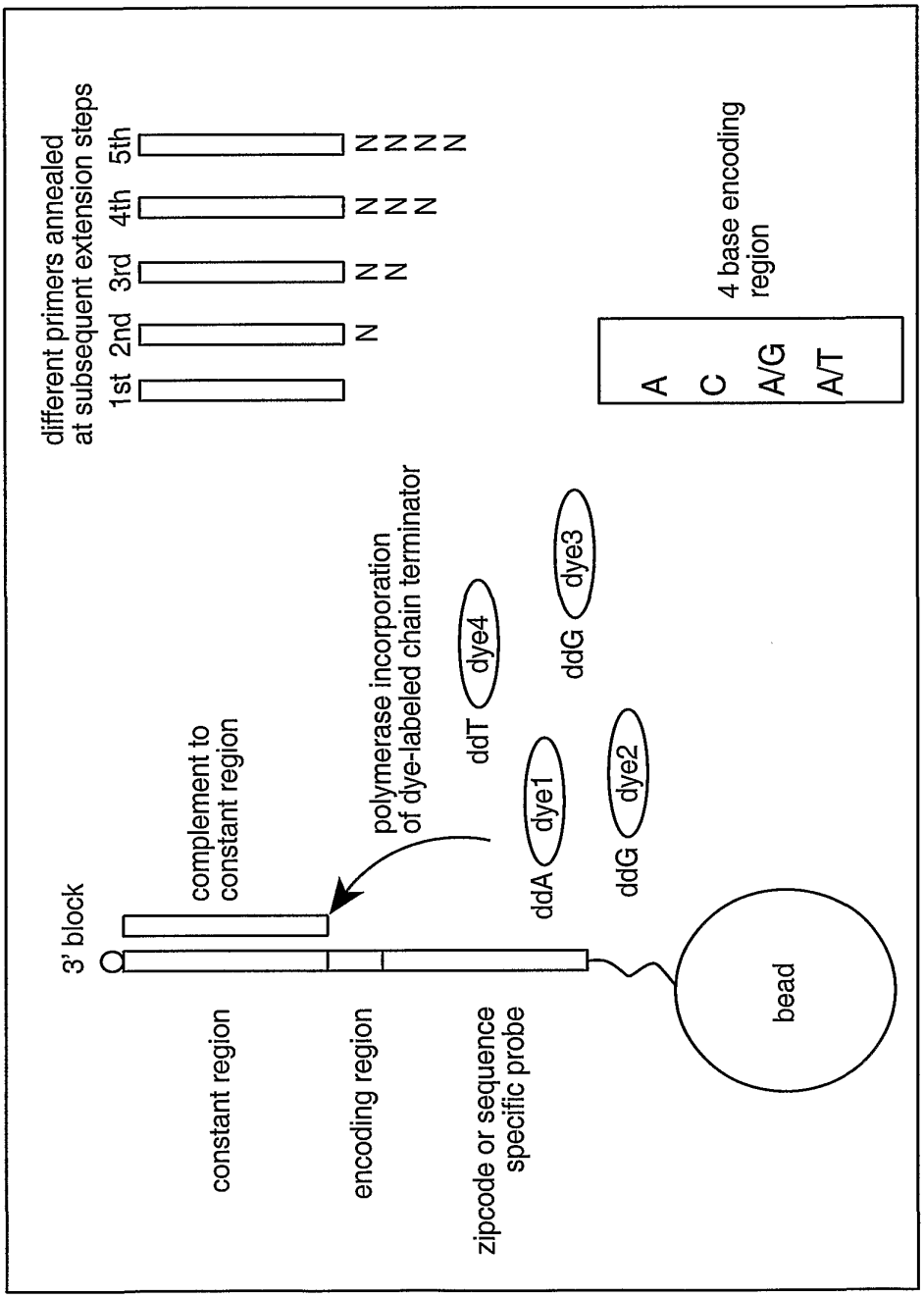


FIG._12

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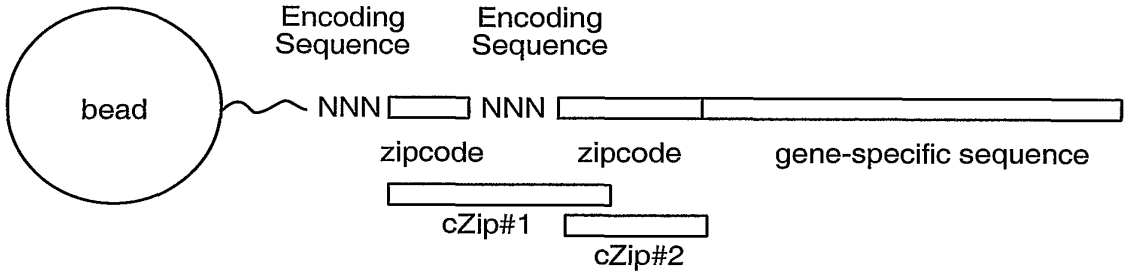


FIG._13

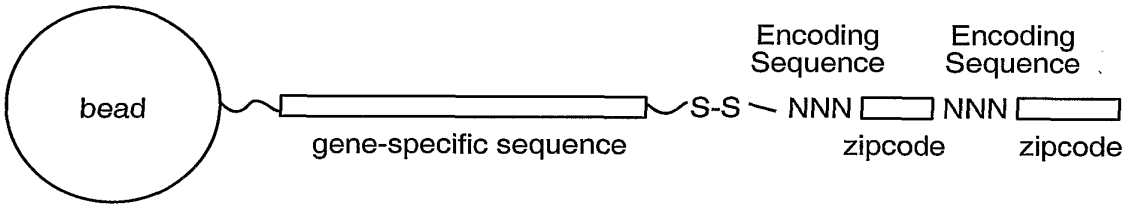


FIG._14

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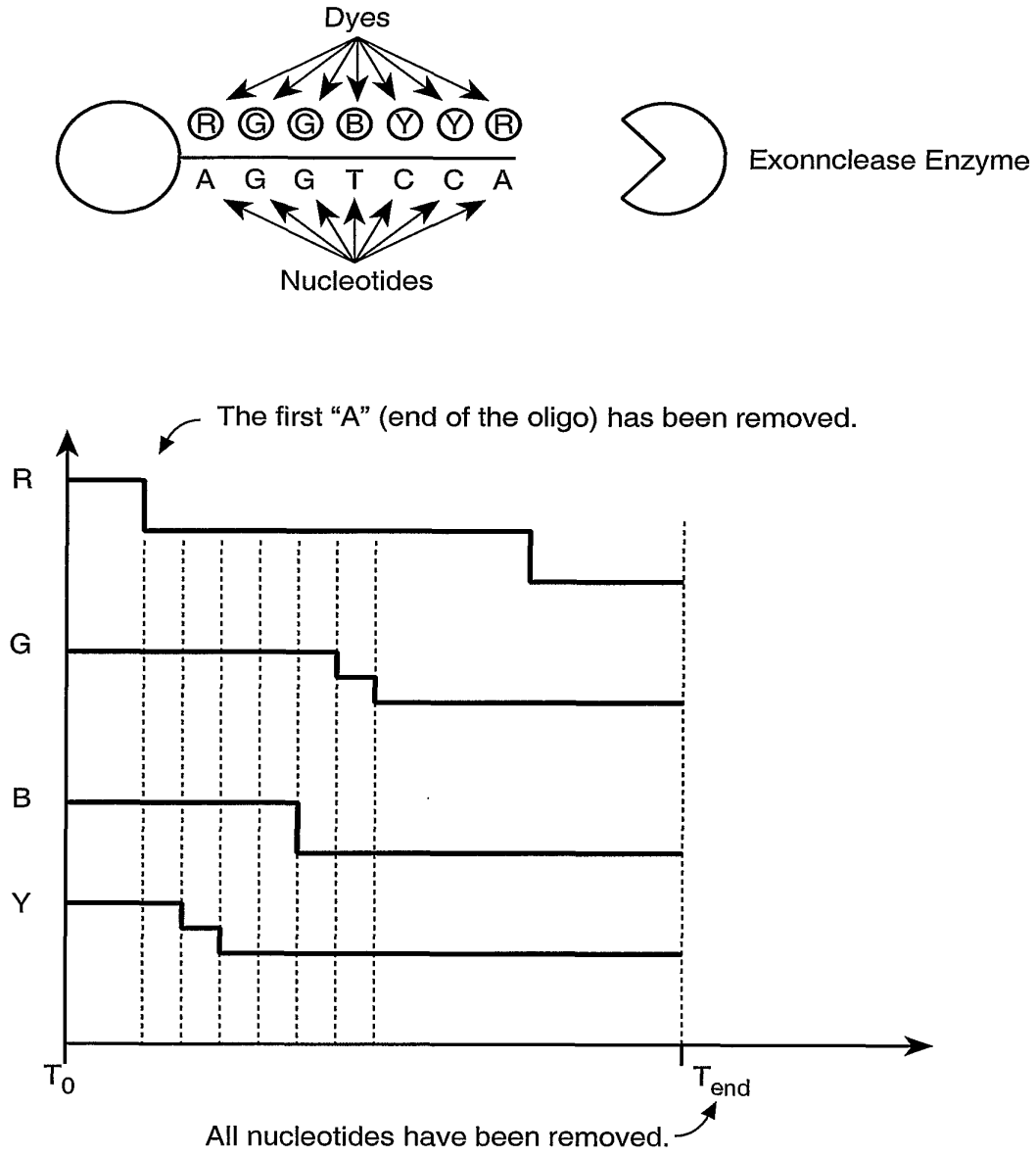


FIG._15

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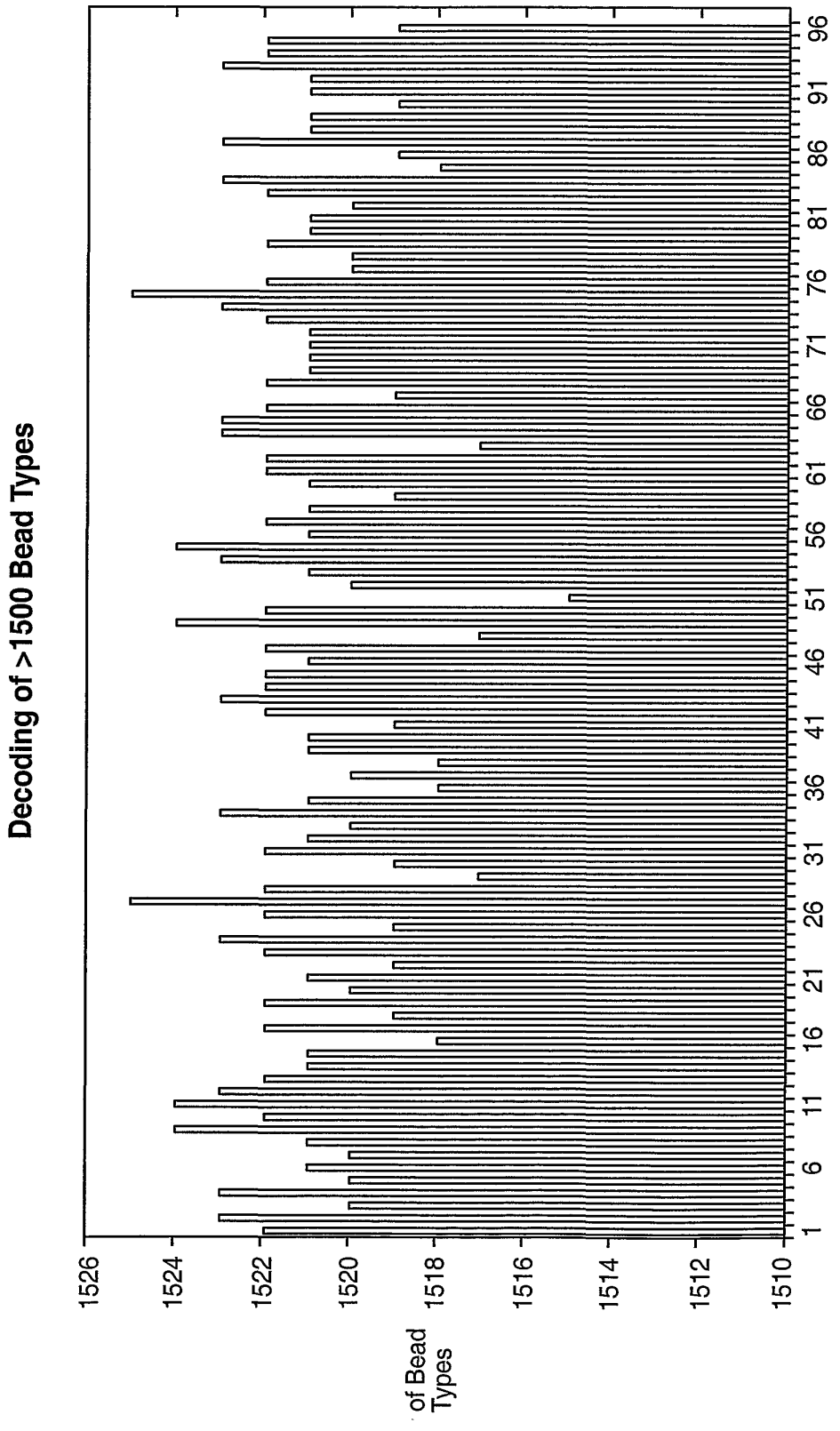


FIG._16

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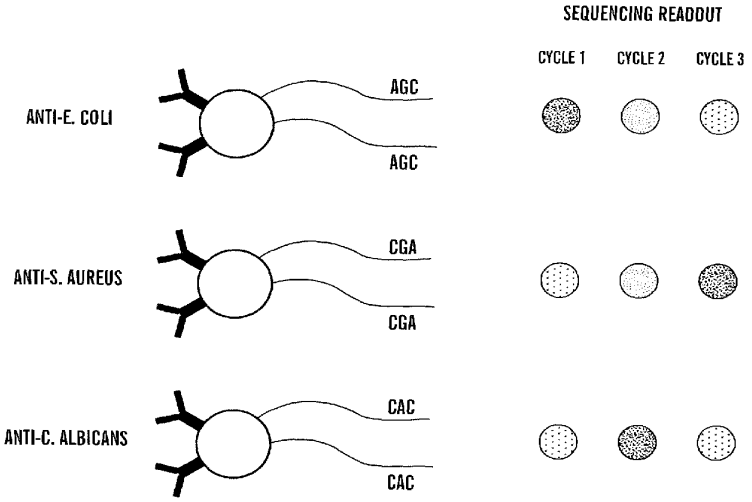
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Published:

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(54) **Title**: COMPOSITIONS AND METHODS FOR ANALYTE DETECTION



(57) **Abstract**: The inventions provided herein relate to detection reagents, compositions, methods, and kits comprising the detection reagents for use in detection, identification, and/or quantification of analytes in a sample. Such detection reagents and methods described herein allow multiplexing of many more labeled species in the same procedure than conventional methods, in which multiplexing is limited by the number of available and practically usable colors.

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COMPOSITIONS AND METHODS FOR ANALYTE DETECTION

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. §119(e) of U.S. Provisional Application No. 61/579,265 filed December 22, 2011, the content of which is incorporated herein by reference in its entirety.

GOVERNMENT SUPPORT

[0002] This invention was made with government support under HG005550 awarded by the National Institutes of Health. The government has certain rights in the invention.

TECHNICAL FIELD OF THE DISCLOSURE

[0003] The inventions provided herein relate to detection molecules or detection reagents, compositions, methods and kits for detection, identification, and/or quantification of analytes in a sample.

BACKGROUND

[0004] The need for multiplexing techniques in biology is often driven by the fact that test samples are precious and those analyzing them either do not know in advance precisely what to look for or must extract the most information from any single sample. Hence, it is desirable for clinicians and researcher to subject each sample to a large set of probes.

[0005] Optical readout is common in biology and can be very effective. However, it is typically limited to a relatively small number of available fluorophores or chromophores (which are referred to collectively as colors). In practice, multiplexing by fluorescence is often limited to 4 or 5 colors, which by traditional methods implies that at most 4 or 5 probes can be detected in a single sample.

[0006] The common approach to improving multiplexing in optical methods is to increase the number of available colors. To this end, quantum dots have been developed to provide a larger range of colors. However, in reality, it is difficult to use more than 6 quantum dot colors simultaneously. Another approach is to use mixtures or ratio of fluorophores as new colors. Such methods have extended multiplexing to hundreds of analytes, but due to the size of the labels (e.g., microbeads), the technology has thus far been limited to flow-cytometry based

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analyses. Yet another approach involves nanostrings, which are essentially short strings of strung-up fluorophores creating visible colorful barcodes. Unfortunately, nanostring readout requires very high-resolution imaging and a special flow apparatus. Further, the nanostrings can only be used in a sample where the probes' targets are sparse, or the barcodes will overlap and create a blur.

[0007] A simple workaround for the limited number of colors (e.g., 4 or 5 colors) in optical readouts is to repeat the probing of the same sample with multiple small sets of different probes. For example, the assay can involve probing the sample with 4 different antibodies at a time and imaging after every assay. If the test requires probing the sample with a total of 64 antibodies, the 4-probe procedure would have to be repeated 16 times using the sample. As such, the order of detecting different target analytes in a single sample may need to be prioritized, because some target analytes in the sample can degrade during successive probings. Accordingly, there is still a strong need for accurate and sensitive methods with a high throughput for detection, identification, and/or quantification of target molecules in a sample, e.g., complex mixtures.

SUMMARY

[0008] Embodiments provided herein are based on, at least in part, the development of a multiplexed biological assay and readout, in which a multitude of detection reagents comprising one or more probes and/or probe types are applied to a sample, allowing the detection reagents to bind target molecules or analytes, which can then be optically identified in a temporally-sequential manner. In some embodiments, the multitude of detection reagents comprising one or more probes and/or probe types can be applied to a sample simultanesouly. Accordingly, provided herein are methods, compositions (e.g., detection reagents) and kits for detecting multiple analytes in a sample.

[0009] Accordingly, one aspect provided herein relates to a method for detecting a plurality of analytes in a sample. Exemplary analytes include, without limitations, antigens, receptors, proteins, peptides, nucleic acids, sugars, lipid, carbohydrates, glycans, glycoproteins, oligonucleotides, cells, viruses, and any combinations thereof. In some embodiments, the nucleic acids can include, e.g., cellular DNA or RNA, messenger RNA, microRNA, ribosomal RNA, and any combinations thereof. A sample amenable to the methods described herein can be a sample from any sources, e.g., but not limited to biological samples, e.g., collected from

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organisms, animals or subjects, environmental samples, food, food byproduct, soil, archaeological samples, extraterrestrial samples, or any combinations thereof. For example, a sample can be a protein sample immobilized on a solid support including, e.g., a blotting membrane. In alternative embodiments, a sample can comprise one or more cells, one or more tissues, one or more fluids, or any combinations thereof. In some embodiments, the sample can comprise a tissue sample. In some embodiments, the sample can comprise a fluid sample. In some embodiments, a sample can comprise blood, sputum, cerebrospinal fluid, urine, saliva, sperm, sweat, mucus, nasal discharge, vaginal fluids or any combinations thereof. In some embodiments, a sample can comprise a biopsy, a surgically removed tissue, a swap, or any combinations thereof.

[0010] The method described herein comprises: (a) contacting the sample with a plurality of detection reagents as described herein, wherein each subpopulation of the detection reagents can target at least one different analyte; and (b) detecting in a temporally-sequential manner said plurality of the pre-determined subsequences of said detection reagents, wherein said detection of the subsequences each generates a signal signature corresponding to said subsequence, and wherein a temporal order of the signal signatures corresponding to said plurality of the subsequences of said detection reagent identifies a subpopulation of the detection reagents. In some embodiments, the temporal order of the signal signatures corresponding to said plurality of the subsequences of said detection reagent can be unique for each subpopulation of the detection reagents.

[0011] In some embodiments, a detection reagent described herein can target at least two distinct analytes. In some embodiments, a first subpopulation of the detection reagents can target at least one analyte different from that of a second subpopulation of the detection reagents. Accordingly, in some embodiments, the readout of the detection reagents can be distinct but overlapping.

[0012] In some embodiments, the method can further comprise processing the sample before contacting with the plurality of detection reagents described herein.

[0013] In some embodiments, the method can further comprise removing any unbound detection reagents before detection of the pre-determined subsequences in a temporally-sequential manner.

[0014] In some embodiments, the method can further comprise comparing the temporal order

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of the signal signatures with different identifiers of said at least one probe reagent, wherein an agreement between the temporal order of the signal signatures and a particular identifier of said at least one probe reagent identifies the analyte in the sample. In some embodiments, the method can further comprise measuring the intensity of the signal signatures generated from each subpopulation of the detection reagents. In some embodiments, the intensity of the signal signatures generated from each subpopulation of the detection reagents can indicate an amount of the analyte. 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 scheme of the detection reagents described herein. The comparing and intensity measuring steps can be performed by a computer-implemented software or algorithm.

[0015] Each signal signature corresponding to individual pre-determined subsequences of the detection reagent are detected in a temporally-sequential manner. In some embodiments, the detection method can include sequencing, e.g., which can be performed via any methods known in the art, including but not limited to, ligation, hybridization, synthesis, amplification, single-base extension, or any methods known in the art. In certain embodiments, the detection method can include hybridizing a decoder probe with the corresponding subsequence, wherein the decoder probe can comprise a detectable label.

[0016] In particular embodiments, the detection method can comprise: (a) hybridizing a set of decoder probes with a subsequence of the detection reagents, wherein each subpopulation of the decoder probes can comprise a detectable label, each detectable label producing a signal signature; (b) detecting said different signal signature produced by the hybridization of said set of decoder probes; (c) optionally removing said different signal signature produced by the hybridization of said set of decoder probes; and (d) repeating steps (a) through (c) for other subsequences of said detection reagents, thereby producing a temporal order of the signal signatures corresponding to said each detection reagent. In some embodiments, removal of the different signal signature produced by the hybridization can be performed by washing, heating, photo-bleaching, displacement (e.g., displacement of decoder probes with another reagent or nucleic acid sequence), cleavage, enzymatic digestion, quenching, chemical degradation, bleaching, oxidation, or any combinations thereof.

[0017] In some embodiments, each decoder probe in the set can independently have a

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subsequence of the detection reagents.

[0018] In some embodiments, each subpopulation of the decoder probes can comprise a different detectable label, each different detectable label producing a different signal signature.

[0019] In some embodiments, each subpopulation of the decoder probes can be complementary (e.g., partially complementary or completely complementary) to the subsequence of the detection reagents. In some embodiments, a first subpopulation and a second subpopulation of the decoder probes can be complementary (e.g., partially complementary or completely complementary) to the same subsequence of the detection reagents. In some embodiments, a first subpopulation and a second subpopulation of the decoder probes can be complementary (e.g., partially complementary or completely complementary) to distinct subsequences of the detection reagents.

[0020] In some embodiments involving decoder probes for detection purposes, the detectable label associated with each subpopulation of the decoder probes can comprise an optical label selected from the group consisting of a small-molecule dye, a fluorescent molecule or protein, a quantum dot, a colorimetric reagent, a chromogenic molecule or protein, a Raman label, a chromophore, and any combinations thereof. In some embodiments, the detectable label or optical label can be a fluorescent molecule or protein.

[0021] Types of signal signature(s) can vary upon different embodiments of detection reagents and/or decoder probes described herein. By way of example, the detection reagents and/or decoder probes can comprise an optical molecule or label, thus producing optical signatures. Examples of optical signatures can include, without limitations, signatures of fluorescent color (e.g., emission spectra under one or more excitation spectra), visible light, no-color or no-light, color (e.g., color defined by a visible light wavelength), Raman signatures, and any combinations thereof. In some embodiments, an optical signature can comprise signatures of one or more fluorescent colors, one or more visible lights, one or more no-colors or no-lights, one or more colors, one or more Raman signatures, or any combinations thereof. For example, in one embodiment, an optical signature can comprise a plurality (e.g., at least 2 or more) of fluorescent colors (e.g., fluorescent dyes). In these embodiments, the optical signatures can be detected by optical imaging or spectroscopy.

[0022] The spatial movement limit of an analyte in a sample allowed for a temporal detection of the detection reagents to occur can vary depending on a number of factors, including, but not

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limited to, presence of any distinguishable features within a field of detection, magnification used in detection (e.g., magnification of the microscope lens), density of the analytes in a sample, and any combinations thereof. In some embodiments, there can be no limit in the spatial movement of an analyte in a sample during a temporal detection of the detection reagents, for example, provided that the analyte stay within the field of detection and there is at least one same distinguishable feature in each image taken during a temporal detection so that the images can be aligned to each other based on the same distinguishable feature. In some embodiments where there is no such distinguishable feature, the spatial movement of an analyte in a sample can be less than 100 μm, including less than 50 μm, less than 25 μm, less than 10 μm, less than 1 μm or smaller, over a time period, during which a temporal detection of the detection reagents occurs. In some embodiments, the spatial movement of an analyte in a sample can be less than 1000 nm, including less than 500 nm, less than 250 nm, less than 100 nm, less than 50 nm, less than 10 nm or smaller, over a time period, during which a temporal detection of the detection reagents occurs. More importantly, the spatial movement limit of an analyte in a sample during a temporal detection is determined by the ability of matching distinguishable features between images taken during a temporal detection, which can be affected by imaging conditions. In some embodiments, the analyte can be fixed on a solid substrate or support.

[0023] In one aspect, embodiments provided herein relate to a detection reagent, which can be used in a multiplexing assay. The detection reagent comprises at least one probe reagent and at least one nucleic acid label, wherein said at least one nucleic acid label comprises at least one pre-determined subsequence to be detected in a temporally-sequential manner; wherein said at least one pre-determined subsequence forms an identifier of said at least one probe reagent; and wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together.

[0024] In some embodiments, the probe reagent and the nucleic acid label can be conjugated together by at least one linker. The linker can be monovalent or multivalent. Exemplary linkers include, but are not limited to, a bond, a linker molecule, and/or a particle, for example, selected from a group consisting of a gold nanoparticle, a magnetic bead or nanoparticle, a polystyrene bead, a nanotube, a nanowire, a microparticle, and any combinations thereof. In some embodiments, the linker can be a nanoparticle. Examples of linker molecules can include, but are not limited to, a polymer, sugar, nucleic acid, peptide, protein, hydrocarbon, lipid, polyethelyne

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glycol, crosslinker, or combination thereof.

[0025] When the linker is a particle, in some embodiments, the particle can be modified by any methods known in the art. For example, the particle can be coated with streptavidin or a derivative thereof. In some embodiments, the particles can be modified or functionalized with at least one functional group. Examples of the functional groups can include, but are not limited to, amine, carboxyl, hydroxyl, aldehyde, ketone, tosyl, silanol, chlorine, hydrazine, hydrazide, photoreactive groups and any combination thereof.

[0026] The probe reagent of the detection reagent can be any targeting molecule of interest. Examples of the probe reagent can include, but are not limited to, a nucleic acid, an antibody or a portion thereof, an antibody-like molecule, an enzyme, a cell, a virus, an antigen, a small molecule, a protein, a peptide, a peptidomimetic, a sugar, a lipid, a glycoprotein, a peptidoglycan, an aptamer, and any combinations thereof. In some embodiments, the probe reagent can be modified by any means known to one of ordinary skill in the art. By way of example, the probe reagent can be genetically modified, or it can be biotinylated.

[0027] The nucleic acid label of the detection reagent can have any configuration and/or any sequence length. In some embodiments, the nucleic acid label can be single-stranded, double-stranded, partially double-stranded, a hairpin, linear, circular, branched, a concatemer (e.g., a concatemer with a 3D structure such as a rolony, i.e., rolling-circle colony, or a DNA nanoball), or any combinations thereof. In various embodiments, the nucleic acid label can be designed for minimal cross-hybridization of bases with each other.

[0028] In some embodiments, the nucleic acid label can be a modified nucleic acid label. An exemplary modification of the nucleic acid label includes, without limitations, conjugation of the nucleic acid label to one or more detectable molecules. The detectable molecule can include any optical molecule, including, but not limited to, a small-molecule dye, a fluorescent protein, a quantum dot, or any combinations thereof.

[0029] In some embodiments, the nucleic acid label can comprise at least a partially double-stranded region. For example, the nucleic acid label can be pre-hybridized with at least one optically-labeled decoder probe, e.g., to produce at least the first signal of the temporal image stack. In such embodiments, additional decoder probes can be added during the detection method described herein to hybridize with other single-strand subsequences of the nucleic acid label.

[0030] In some embodiments, the nucleic acid label can comprise a plurality of pre-

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determined subsequences. Each of the pre-determined subsequences can be independently of any length. In some embodiments, at least one of the pre-determined subsequences can comprise one or more nucleobases. In certain embodiments, at least one of the pre-determined subsequences can comprise from 1 to 100 nucleobases.

[0031] The pre-determined subsequences with the nucleic acid label can be conjugated together by at least one sequence linker. In some embodiments, the sequence linker can be a direct bond, e.g., a phosphoester bond, which can allow conjugation of the pre-determined subsequences to form a longer, contiguous sequence. In some embodiments, the sequence linker can be a nucleotidic linker. When the pre-determined subsequences are conjugated together by a nucleotidic linker, the nucleotidic linker can have a sequence length of at least one nucleotide. The nucleotidic linker, in some embodiments, can be single-stranded, double-stranded, partially double-stranded, a hairpin, or any combinations thereof.

[0032] Depending on various applications and/or assay conditions (e.g., sensitivity, sample volume/concentration), a detection signal of a probe can be amplified by conjugating the probe to a plurality of the nucleic acid labels. In such embodiments, the detection reagent can comprise one probe reagent and a plurality of nucleic acid labels. Without wishing to be limiting, in other embodiments, the detection reagent can comprise a plurality of probe reagents and a nucleic acid label. In some embodiments, the detection reagent can comprise a plurality of probe reagents and a plurality of nucleic acid labels.

[0033] The detection reagents and methods described herein can be used in any biological assays for detection, identification and/or quantification of target molecules or analytes in a sample. In particular embodiments, the detection reagent can be present in a soluble phase for various biological assays. By way of example only, in some embodiments, the detection reagent can be adapted for use in immunofluorescence. For example, the detection reagent adapted for use in immunofluorescence can be used to identify microbes or pathogens. In alternative embodiments, the detection reagent can be adapted for use in immunohistochemistry. For example, the detection reagent adapted for use in immunohistochemistry can be used to study tissue biopsies or cultured cells. In some embodiments, the detection reagent and the method described herein can be applied to fixed cells and/or living cells. In other embodiments, the detection reagent can be adapted for use in fluorescence in situ hybridization. In some embodiments, the detection reagent can be adapted for use in western blot. Accordingly, the

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detection reagent described herein can be adapted for use in various applications, e.g., but not limited to, pathogen detection and/or identification, cancer-tissue analysis and other medical pathology applications, lineage tracking of differentiating stem cells, and lineage tracking and/or identification of dendritic cells.

[0034] Kits for various biological assays also provided herein. In some embodiments, a kit can comprise: (a) a plurality of the detection reagents described herein or a portion thereof; and (b) at least one reagent. Examples of a reagent include, but are not limited to, a readout reagent, a wash buffer, a signal removal buffer, and any combinations thereof.

[0035] The kits provided herein can be used for sequencing-based readout or hybridization-based readout. In some embodiments where the kit is used for hybridization-based readout, the kit can further comprise at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.

[0036] In some embodiments, the kit can comprise a plurality of at least one component of the detection reagents, e.g., the "nucleic acid label" component of the detection reagents. In such embodiments, users can attach the provided nucleic acid labels to their probe reagents of interest to form their own detection reagents described herein. In such embodiments, the kit can further comprising at least one coupling agent that allows the user to conjugate at least one nucleic acid label to the user's probe reagents of interest. In other embodiments, the nucleic acid labels can be already attached to the pre-determined probe reagents and are thus provided to users in the form of the detection reagents that are ready to use.

[0037] In some embodiments, the detection reagents provided in the kit or formed by a user can be provided in a solution phase. In other embodiments, the detection reagents provided in the kit or formed by a user can be immobilized in a multi-well plate.

[0038] In some embodiments of any aspects described herein, the analytes or target molecules can be present in a solution phase. In some embodiments, the analytes or target molecules can be immobilized on a solid substrate or support.

[0039] To clarify, the compositions and methods described herein are different from the ones described in the US Patent Application No.: US 2007/0231824. The '824 application discusses methods of decoding a sensor array containing immobilized microspheres, wherein the

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microspheres are immobilized on a solid support (e.g., an array substrate), rather than designed to be in a solution phase. As such, a sample fluid is flowed over the sensor array containing immobilized microspheres. The analytes in the sample fluid then bind to the immobilized microspheres. After binding, the sample fluid is then discarded and the immobilized microspheres are analyzed. Accordingly, the compositions and the methods described in the '824 application cannot be used and detected directly on a sample (e.g., on a tissue sample) or in situ as described herein, e.g., immunofluorescence, immunohistochemistry, fluorescence in situ hybridization, or western blot.

[0040] Further, the assay methods described herein are also different from the general nanostring technology or other technologies as described in the U.S. Patent No.: US 7,473,767, and the U.S. Patent Application No.: US 2010/0047924. The general nanostring technology and nanoreporter detection methods described in the '924 application are based on determination of the "spatial location of signals" emanating from the labeled nanostrings or nanoreporters. The detection methods described in the '767 patent is based on the color resulting from various ratios of different optical labels bound to the polynucleotide probes. All these previous methods will require at least a plurality of optical labels to be detected simultaneously for determination of spatial location of signals or the resultant color from various ratios of different optical labels. Accordingly, all these previous methods do not involve detection of signals in a temporally-sequential manner as described herein. Further, there are at least two disadvantages of nanostring technology based on detection of "spatial location of signals," rather than temporal detection of signals as described herein. First, compared to the methods and detection reagents described herein, the nanostring technology generally requires very high optical magnification for spatially discerning separation of colors that are typically located very close to each other within a nanostring; thus limiting a field of view/sample size, and precision, and/or increasing instrument cost. Second, unlike the methods and detection reagents described herein, the nanostring technology generally requires a thorough control of the amount of probes used in detection, because too few probes would yield a signal that is difficult to be detected, but too many probes would increase the likelihood of probes overlapping, and thus making the readout impossible.

BRIEF DESCRIPTION OF THE DRAWINGS

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[0041] **Fig. 1** shows three different embodiments of the detection reagents described herein producing distinct sequencing readout. Pathogen-specific antibodies (e.g., anti-E-Coli, anti-S. aureus, and anti-*C. albicans*) are individually conjugated to a nanoparticle with at least one nucleic acid label as described herein. In accordance with one or more embodiments, the readout of the nucleic acid label can take the form of a set of optical images or spot-readings of, e.g., fluorescent or visible colors; the temporal sequence of optical images or spot-readings can then be computationally analyzed to determine the identity of the corresponding probe reagent, e.g., a pathogen-specific antibody.

[0042] **Fig. 2** shows three different embodiments of the detection reagents described herein producing distinct sequencing readout. DNA oligonucleotides complementary to target RNA expression (e.g., OCT4, SOX2, or KLF4) are individually conjugated to a nanoparticle with at least one nucleic acid label as described herein. In accordance with one or more embodiments, the readout of the nucleic acid label can take the form of a set of optical images or spot-readings of, e.g., fluorescent or visible colors; the temporal sequence of optical images or spot-readings can then be computationally analyzed to determine the identity of the corresponding probe reagent, e.g., a DNA aptamer specific for a RNA expression.

[0043] **Fig. 3** shows exemplary forms of a nucleic acid label of the detection reagent according to one or more embodiments described herein.

[0044] **Figs. 4A** and **4B** shows two exemplary configurations of probe reagents and nucleic acid labels on particles, in accordance with one or more embodiments described herein.

[0045] **Fig. 5** shows one embodiment of the detection reagents for an exemplary hybridization-based readout method, in accordance with one or more embodiments described herein. Pathogen-specific antibodies (e.g., anti-*C. albicans*) are individually conjugated to a nanoparticle comprising at least one nucleic acid label as described herein. In accordance with one or more embodiments, the readout of the nucleic acid label can be determined by hybridizing it with a small number of, e.g., fluorescently-labeled decoder probes, imaging, and then advancing to the next set of decoder probes. In order to allow the SeqTag to be read out quickly and without the use of enzymes or chemical reactions, the DNA oligonucleotide (SeqTag) is designed to include several hybridization sites, each corresponding to a particular readout step. At each readout step, the sample is subjected to a mixture of fluorescently labeled DNA probes that could potentially bind that step's hybridization site. Each of the sites, however, is designed

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to bind only one of these probes, thus revealing the SeqTag's identifying code.

[0046] **Figs. 6A-6B** show the readout images of DYNABEADS® beads (1 μm in size) localized on a sample using an exemplary hybridization-based detection method. Each bead was conjugated to one of 6 nucleic acid labels, which in turn hybridized with a different set of decoder probes conjugated to either a green, red or blank optical label during each readout stage (**Fig. 6A**: Readout stage 1; **Fig. 6B**: Readout stage 2).

[0047] **Fig. 7** is a set of images showing that each of the detection molecule constructs (SeqTag labels) properly stained the yeast and fluorescenced in accordance with three pre-determined sets of decoder probes. SeqTag labels and oligonucleotide displacers were applied as follow:

Step 1: Set 1 Probes only;

Step 2: Set 1 Displacement oligonucleotides and Set 2 Probes;

Step 3: Set 2 Displacement oligonucleotides and Set 3 Probes; and

Step 4: Set 3 Displacement oligonucleotides.

SeqTag Label and corresponding colors were as follow:

Set 1: SeqTag 1 - Green, SeqTag 2 - Red, and SeqTag 3 - Blue;

Set 2: SeqTag 4 - Green, SeqTag 5 - Red, and SeqTag 6 - Blue;

Set 3: SeqTag 7 - Green and SeqTag 8 - Red.

[0048] **Fig. 8** shows SeqTag readout by a displacement-hybridization experiment according to an embodiment of the method described herein. SeqTag DNA labels were incubated on a streptavidin-coated microarray. This microarray was then exposed to a sequence of fluorescently labeled detection probes and displacement oligonucleotidess, as per displacement-hybridization readout. Imaging the array after each readout step demonstrated fluorescence corresponding to the expected pattern (shown next to each image).

[0049] **Fig. 9** is a schematic representation of displacement hybridization according to an embodiment of the method. As the readout progresses, fluorescence from prior readout steps needst be removed so as not to obstruct the current step's signal. As illustrated in **Fig. 9**, this can be accomplished using a displacement-hybridization method: each of the SeqTag's hybridization sites can be preceded by a short "toehold" sequence. During each readout step, the sample is subjected to a mixture of "displacer" DNA oligonucleotides. One of these displacers is

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complementary to the preceding step's hybridization site and, with the help of the toehold, is capable of displacing the fluorescent probe that was bound there.

[0050] **Fig. 10** is a schematic representation of a probe reagent according to an embodiment described herein. Shown is an oligonucleotide-antibody-streptavidin construct. A convenient and effective way to SeqTag-label antibodies is through a streptavidin bridge. The antibody is biotinylated and the probe DNA oligonucleotides (SeqTag) are synthesized with a 5'-biotin modification. Then, by taking advantage of streptavidin' s native tetrameric form, three DNA strands are bound to each antibody. As opposed to chemical conjugation methods, which can harm the antibody, this method proves gentle enough to preserve antibody function. As shown a single streptavidin molecule (2) is bound to three of the same DNA SeqTags (1) and a single antibody (3).

[0051] **Fig. 11** is a schematic representation of detection of an analyte by the SeqTag labeled antibody shown in **Fig. 10**.

[0052] **Fig. 12** is a schematic representation of detection reagents for different analytes. As shown, different infectious agent (analytes) can each be assigned their own SeqTag code to enable multiplexed detection according to an embodiment described herein.

[0053] **Fig. 13** is a schematic representation of detection reagents for SeqTagged Fluorescence *in situ* hybridization (FISH). FISH permits microbes to be identified based on their ribosomal RNA sequence. In the case of SeqTagged FISH, the FISH probe and its SeqTag can be synthesized as a single DNA oligonucleotides as shown. Sequences shown are, from top to bottom, SEQ ID NO: 1 (5'-CCTACACACCAGCGTGCC-3', probe for *K. pneumonia*); SEQ ID NO: 2 (5'-CCGCACTTTCATCTTCCG-3', probe for *H. influenza*); and SEQ ID NO: 3 (GCCAAGGCTTATACTCGC, probe for *C. albicans*).

DETAILED DESCRIPTION OF THE INVENTION

[0054] Described herein are methods, detection reagents (or detection molecules as used interchangeably herein) and kits for detecting a plurality of analytes in a sample. In accordance with embodiments of various aspects described herein, a probe reagent (e.g., antibody or aptamers) can be directly or indirectly labeled with a nucleic acid label. The nucleic acid information present on the nucleic acid label can then be decoded and/or detected in a temporally-sequential manner. The detection reagents and methods described herein significantly

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increase the number of different probes (and corresponding analytes) that can be simultaneously detected in a multiplex assay, as compared to an traditional assay where each probe is labeled with only fluorescent labels or quantum dots, and thus multiplexing is limited by the number of available and practically usable colors. Furthermore, because the detection reagents described herein are detected and/or imaged in a temporal series of steps, the number of probes (and corresponding analytes) that can be detected in a multiplex assay grows multiplicatively with the number of detection steps in a time series and the number of optical labels being used. By way of example only, 3 set of images in which 4 distinct optical labels are used can encode 4 x 4 x 4 = 64 distinct probe reagents (e.g., antibodies).

Methods of detecting a plurality of analytes in a sample

[0055] One aspect of the inventions provides the methods for detecting a plurality of analytes in a sample, using the detection reagents described herein. The method includes (a) contacting the sample with a composition comprising a plurality of detection reagents (which will be described in detail later), wherein each subpopulation of the detection reagents targets at least one different analyte; and (b) detecting in a temporally-sequential manner said plurality of the pre-determined subsequences of said detection reagents, wherein said detection of the subsequences each generates a signal signature corresponding to said subsequence, and wherein a temporal order of the signal signatures corresponding to said plurality of the subsequences of said detection reagent identifies a subpopulation of the detection reagents. In some embodiments, the signal signature is a temporal signature. In some embodiments, the signal signature can further comprise a spatial signature. A non-limiting example of a spatial signature includes spatial location of a signal signature.

[0056] In some embodiments, the temporal order of the signal signatures corresponding to the plurality of the subsequences of the detection reagent can be unique for each subpopulation of the detection reagents. In some embodiments, at least two or more signal signatures can be used to identify the same subpopulation of the detection reagents.

[0057] In some embodiments, a detection reagent described herein can target at least two (e.g., at least two, at least three or more) distinct analytes. In some embodiments, a first subpopulation of the detection reagents can target at least one analyte different from that of a second subpopulation of the detection reagents. By way of example only, a first subpopulation of

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the detection reagents can target at least analyte A and analyte B, whereas a second subpopulation of the detection reagents can target at least analyate B and analyte C. The readout of these detection reagents can be distinct but overlapping. Thus, different analytes can be identified by sampling them combinatorially and determining which one binds.

[0058] As used herein, the term "temporal order of the signal signatures" refers to a sequence of signal signatures determined in a temporally-sequential manner, i.e., the sequence of signal signatures is progressed through by a number of active operations performed in a temporally-sequential manner, e.g., using a different set of decoding reagents or decoder probes in each active operation. In some embodiments, using a set of decoder probes in each active operation can yield one signal signature corresponding to one subsequence of the detection reagents.

[0059] The composition comprising a plurality of detection reagents can exist in any format. In some embodiments, the composition comprising a plurality of detection reagents can be in a form of a solution or suspension comprising the detection reagents. In such embodiments, the composition can further comprise at least one agent. For example, without wishing to be bound, the agent can be a blocking buffer, a surfactant, unconjugated probe reagents, a stabilizer, an enzyme inhibitor, or any combinations thereof. In some embodiments where the compositions are administered *in vivo*, the composition can further comprise a pharmaceutically-acceptable carrier. In other embodiments, the composition comprising a plurality of detection reagents can be contained or immobilized in a device (e.g., a syringe, or a microfluidic device) or an assay or reaction vessel (e.g., solid supports such as vials, and multi-well plates).

[0060] As used therein, the term "contacting" refers to any suitable means for delivering, or exposing, a sample to a plurality of the detection reagents described herein. In some embodiments, the term "contacting" refers to adding the detection reagents (e.g., suspended in a solution) directly to the sample. In some embodiments, the term "contacting" can further comprise mixing the sample with the detection reagents by any means known in the art (e.g., vortexing, pipetting, and/or agitating). In some embodiments, the term "contacting" can further comprise incubating the sample together with the detection reagents for a sufficient amount of time, e.g., to allow binding of the probe reagents to the target analytes. The contact time can be of any length, depending on the binding affinities and/or concentrations of the probe reagents and/or the analytes, concentrations of the detection reagents, and/or incubation condition (e.g., temperature). For example, the contact time can be reduced if the sample and detection reagents

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are incubated at a higher temperature. In some embodiments, the contact time between the sample and the detection reagents can be at least about 30 seconds, at least about 1 minute, at least about 5 minutes, at least about 10 minutes, at least about 15 minutes, at least about 30 minutes, at least about 1 hour, at least about 2 hours, at least about 3 hours, at least about 4 hours, at least about 6 hours, at least about 8 hours, at least about 10 hours, at least about 12 hours, at least about 24 hours, at least about 48 hours or longer. One of skill in the art can adjust the contact time accordingly.

[0061] For in vivo applications, the term "contacting" can refer to administering the detection reagents to a subject, e.g., by oral administration or by injection.

[0062] The sample can be contacted with at least one kind of the detection reagents. In some embodiments, the sample can be contacted with at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100 or more different kinds of the detection reagents. In some embodiments, the sample can be contacted with at least 100, at least 500, at least 1000, at least 5000, at least 10,000, at least 50,000, at least 100,000 or more different kinds of the detection reagents. Various kinds of the detection reagents described herein can differ in types of probe reagents (e.g., nucleic acids vs. antibodies), target binding domains, and/or target analytes.

[0063] In some embodiments, the method described herein can further comprise processing the sample before contacting with the composition comprising a plurality of detection reagents described herein. Depending on the types and/or natures of the samples and/or analytes, different sample processing techniques can be used with the methods described herein. Exemplary sample processing techniques include, but are not limited to, mechanical processing of a sample (e.g., without limitations, homogenizing, centrifuging, vortexing, sectioning and shearing), addition of at least one reagent to a sample (e.g., without limitations, lysis buffers, RNA or DNA extraction reagents, RNA or DNA digestion reagents, enzyme inhibitors, fixing agents, organic solvents, antibodies, permeabilizing agents and immunohistochemistry agents), separation of a sample (e.g., without limitations, filtering, centrifuging, electrophoresis, western blot, and Northern blot), mounting a sample on a solid support (e.g., a microscopic slide), and any combinations thereof.

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[0064] By way of example only, if a sample is a tissue from a subject (e.g., a biopsy for immunostaining), sample processing can include, but are not limited to, tissue sectioning, mounting on a solid support, fixing the tissue, permeabilizing the tissue (if intracellular proteins are to be detected), blocking non-specific reactions with the detection reagents. In some embodiments, proteins or nucleic acids can be isolated from a tissue or fluid sample and then separated electrophoretically on a separation medium (e.g., electrophoresis gel), followed by transferring the proteins or nucleic acids to a blotting membrane. The blotting membranes containing proteins or nucleic acids can then be contacted with the detection reagents described herein. Methods of processing samples before addition of various types of probe reagents for different kinds of assays are well established in the art, and any of those methods can be performed prior to the contacting step of the methods described herein.

[0065] In some embodiments, the method described herein can further comprise removing any unbound detection reagents before detection of the pre-determined subsequences in a temporally-sequential manner. The term "unbound detection reagents" as used herein refers to detection reagents that have not bound to or interacted with target analytes. The unbound detection reagents can be removed from the sample by any methods known in the art, e.g., rinsing with the sample with a buffered solution at least once, at least two times, at least three times or above.

[0066] After the detection reagents bind to the target analytes in a sample, the nucleic acid labels of the detection reagents carrying nucleic-acid information can be decoded to allow identification of respective probe reagent(s) conjugated to them, as opposed to traditional optical labeling technologies, where an optical signature such as a fluorophore is detected in the absence of providing any nucleic acid information. In embodiments of various aspects described herein, the pre-determined subsequences within the nucleic acid labels are detected in a temporally-sequential manner. The term "temporally-sequential manner" is used in reference to detecting or decoding in a time series a plurality of the pre-determined subsequences within the nucleic acid labels of any detection reagents that are bound to target analytes in a sample. In some embodiments, one or more pre-determined subsequences within at least one nucleic acid label of each detection reagent can be detected or decoded at each time point or detection step of a time series. In some embodiments, one pre-determined subsequence within at least one nucleic acid label of each detection reagent can be detected or decoded at each time point or detection step of

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a time series. In some embodiments, at least one pre-determined subsequence (e.g., 1, 2, 3, 4, 5, 6, or more pre-determined subsequences) at the same corresponding location within the nucleic acid label of each detection reagent can be detected or decoded at each time point or detection step of a time series. The time period between any two time points or detection steps can be of any length, e.g., seconds, minutes and hours. For example, the time period between any two time points or detection steps can vary from about 5 seconds to about 2 hours, from about 10 seconds to about 1 hour, from about 30 seconds to about 30 mins, or from about 1 min to about 15 mins. In some embodiments, the time period between any two time points or detection steps can be less than 5 seconds. In other embodiments, the time period between any two time points or detection steps can be longer than 2 hours, longer than 4 hours, longer than 6 hours, longer than 12 hours, longer than 1 day. For example, a sample containing the detection reagents can be maintained at room temperature, at a fridge temperature (e.g., between about 0 °C and about 10°C) or at sub-zero temperatures (e.g., between -80°C or lower and 0°C) during the time period between detection steps. In some embodiments, each subsequent detection step is performed substantially immediately one after another (e.g., within less than 2 seconds, less than 1 second).

[0067] In some embodiments, the pre-determined subsequences can be detected in any temporal orders. In some embodiments, the next pre-determined subsequence to be detected after the previous one can be located closest to the previous one. In some embodiments, the next pre-determined subsequence to be detected after the previous one can be located at least one, at least two, at least three, at least four, at least five or more pre-determined subsequences apart from the previous one. In such embodiments, any pre-determined subsequences that were bypassed in a previous detection step can be detected afterward. In some embodiments of the methods described herein, a computer-implemented software can be used to facilitate an analysis of the temporal readouts from the detection steps, e.g., re-arranging the temporal readouts in an order corresponding to their spatial locations within the nucleic acid label before further comparison and quantification analyses.

[0068] In some embodiments, the detection or decoding of the pre-determined subsequences can comprise nucleic acid sequencing. Methods for sequencing nucleic acids are well established to a skilled artisan, e.g., but not limited to ligation, hybridization, synthesis, amplification or single-base extension, or any combinations thereof. By way of example only, as shown in **Fig. 1** or **Fig. 2**, the nucleic acid labels each contain three pre-determined subsequences (each of one

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nucleotide) conjugated together by a direct bond such as a phosphodiester bond. Each sequencing step decodes or determines one nucleotide, wherein each nucleobase (A, G, C or T) generates a distinct signal signature corresponding to the nucleobase. Consequently, a temporal order or time series of the signal signatures generated from each sequencing step corresponds to the respective probe reagent, and thus identify the target analyte. Without wishing to be bound, in this embodiment, the number of sequencing steps performed is not necessarily equal to the number of the pre-determined sequences. In some embodiments, the number of sequencing steps performed can be less than the number of the pre-determined sequences. For example, as shown in **Fig. 1**, two sequencing steps could be sufficient to identify the three different probe reagents, where each base is associated with a different color. However, additional nucleic acid information can increase the accuracy of identifying different probe reagents. Further, if the sequencing of each base can yield one of 4 colors, the n-base subsequences (e.g., 3-base subsequences shown in **Fig. 1** or **Fig. 2**) can produce 4^n possible unique readouts, i.e., 4^n possible distinct probe reagents can be distinguished using such detection reagents described herein.

[0069] While sequencing methods can convey single base difference, other detection or decoding methods that convey information by the presence or absence of entire hybridization "sites" or pre-determined subsequences on the nucleic acid label can also be used for the methods described herein. In some embodiments, the detection step can comprise hybridizing a decoder probe with a subsequence on the nucleic acid label of the detection reagent, wherein the decoder probe can comprise a detectable label. In particular embodiments, the detection method can comprise: (a) hybridizing a set of decoder probes with a subsequence of the detection reagents, wherein each subpopulation of the decoder probes can comprise a detectable label, each detectable label producing a signal signature; (b) detecting said signal signature produced by the hybridization of said set of decoder probes; and (d) repeating steps (a) and (b) for other subsequences of said detection reagents.

[0070] In some embodiments, each subpopulation of the decoder probes can comprise a different detectable label, each different detectable label producing a different signal signature. In these embodiments, the different signal signature produced by the hybridization of the set of decoder probes can be detected.

[0071] In some embodiments, each subpopulation of the decoder probes can be complementary (e.g., partially complementary or completely complementary) to the subsequence

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of the detection reagents. In some embodiments, a first subpopulation and a second subpopulation of the decoder probes can be complementary (e.g., partially complementary or completely complementary) to distinct subsequences of the detection reagents. In some embodiments, at least two or more subpopulations of the decoder probes can bind to the same subsequence of the detection reagents. For example, a first subpopulation and a second subpopulation of the decoder probes can be complementary (e.g., partially complementary or completely complementary) to the same subsequence of the detection reagents.

[0072] By way of example only, **Fig. 5** shows an exemplary detection reagent comprising anti-*C. albicans* probe reagents and nucleic acid labels containing three hybridization sites or pre-determined subsequences conjugated together by sequence linkers. In the first hybridization step, a first set of decoder probes each comprising a distinct detectable label (e.g., complementary DNA readout probes shown in **Fig. 5**, each comprising a distinct optical label) is hybridized with a first pre-determined subsequence (e.g., Site 1 in **Fig. 5**), followed by detection of a first signal signature produced by the hybridization. In the second hybridization step, a second set of decoder probes each comprising a distinct detectable label is hybridized with a second pre-determined subsequence (e.g., Site 2 in **Fig. 5**), followed by detection of a second signal signature produced by the hybridization. The second pre-determined subsequence can be the same or different from the first pre-determined subsequence. However, in preferred embodiments, the second pre-determined subsequence is different from the first pre-determined subsequence, e.g., to minimize cross-hybridization with each other. Accordingly, the hybridization and signal detection steps are repeated for other subsequences of the detection reagents with a different set of decoder probes, thereby producing a temporal order or time series of the signal signatures corresponding to the respective probe reagent (and detection reagent).

[0073] As used herein, the term “decoder probe” refers an oligonucleotide with a sequence complementary to a pre-determined sequence of the nucleic acid label. By "complementary" is meant that a nucleic acid can form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non- traditional types. The decoder probe sequence can be completely or partially complementary to a pre-determined sequence. In some embodiments, partial complementarity is indicated by the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9,10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100%

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complementary). "completely complementary" or 100% complementarity means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. Less than perfect complementarity refers to the situation in which some, but not all, nucleoside units of two strands can hydrogen bond with each other.

[0074] The decoder probe can have a sequence of any length. In some embodiments, the decoder probe can have a sequence length of about 1 to about 100 nucleotides, about 1 to about 50 nucleotides, about 2 to about 50 nucleotides, about 5 to about 30 nucleotides, or about 5 to about 20 nucleotides.

[0075] In some embodiments, the decoder probe can comprise at least one detectable label described herein. In some embodiments, the detectable label can be an optical label selected from the group consisting of a small-molecule dye, a fluorescent molecule or protein, a quantum dot, a colorimetric reagent, a chromogenic molecule or protein, a Raman label, and any combinations thereof. In some embodiments, the detectable label or optical label can be a fluorescent molecule or protein.

[0076] In some embodiments, the decoder probe can be modified, e.g., base modification or activated with a functional group for linkage to a detectable label.

[0077] The number of decoder probes in each set can vary, depending on the number of distinct subsequences in each hybridization. In some embodiments, there can be about 1 to about 100 decoder probes, about 2 to about 50 decoder probes, about 4 to about 20 decoder probes in each set. In some embodiments, there can be about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50 or more decoder probes in each set. In some embodiments, while each subsequence site can hybridize with a large number of decoder probes, each *set* of decoder probes added in each readout step to hybridize with the subsequence site within the detection reagents can generally have as many as the number of available fluorescent colors. For example, each set of the decoder probes added in each readout step to hybridize with the subsequence site of the detection reagents can contain about 3-4 decoder probes, each of which is labeled with a distinct fluorescent color. In the case of using quantum dots or Raman labels as detection labels, there can be more than 3-4 decoder probes in each set added during each readout step.

[0078] Without wishing to be bound, an example of "non-overlapping nucleic acid labels" or "non-overlapping SeqTag labels" (i.e., no two decoder probes will hybridize with the same

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spatial site of the nucleic acid label) is shown herein for illustrative purposes. Assuming there are 12 pre-determined subsequences (e.g., nucleic acid sequences) designed for minimal cross-hybridization with each other and each others' complements (e.g., A1, A2, A3, A4, B1, B2, B3, B4, C1, C2, C3, and C4). Consider a detection reagent comprising a nucleic acid label of the form:

5' ------A[1-4]----B[1-4]----C[1-4]---- 3'

where each position (e.g., A[1-4]) holds only one of the subsequences (e.g., A2). Now, this subsequence is decoded or detected by using decoder probes or complementary probes A1* - A4* each labeled in one of four fluorescent colors. After readout, the signal produced by the fluorophore can be removed (e.g., denaturing to undo the hybridization (and thus removing the fluorophore)) before continuing with B1* - B4*. Since each step yields one of four outcomes, this coding scheme can provide 4 x 4 x 4 = 64 unique readouts. These hybridization-based probes can be used to label 64 different probe reagents that can be read out in three cycles.

[0079] In the case where two decoder probes can overlap, a single site (e.g., A in the above nucleic acid label form), which can accept one of the different decoder probes, can be used. This single site can be read out by subjecting it to the different decoder probes, e.g., in sets of 4 using the nucleic acid label form as shown above. When the correct set is reached, the nucleic acid label, e.g., SeqTag label, should be dark (no-color). This example is not construed to be limiting and any modifications apparent to one of skill in the art is also within the scope of the inventions.

[0080] The advantage of readout by hybridization is that it can be quick: hybridization can take place in minutes or less. Furthermore, no chemistry or enzymes are required during the readout process, as the readout process can be performed by similar methods as used in nucleic acid sequencing, microscopy, spectroscopy, or any combinations thereof. Thus, hybridization-based readout method can reduce cost, reduce reagent storage and/or simplify the process.

[0081] In some embodiments of the methods described herein, there can be no limit in the spatial movement of an analyte in a sample during a temporal detection of the detection reagents, for example, provided that the analyte stay within the field of detection and there is at least one same distinguishable feature in each image taken during a temporal detection so that the images can be aligned to each other based on the same distinguishable feature. In some embodiments where there is no such distinguishable feature, the spatial movement of an analyte in a sample can be less than 100 μm, including less than 50 μm, less than 25 μm, less than 10 μm, less than 1

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μm or smaller, over a time period, during which a temporal detection of the detection reagents occurs. In some embodiments, the spatial movement of an analyte in a sample can be less than 1000 nm, including less than 500 nm, less than 250 nm, less than 100 nm, less than 50 nm, less than 10 nm or smaller, over a time period, during which a temporal detection of the detection reagents occurs. More importantly, the spatial movement limit of an analyte in a sample during a temporal detection is determined by the ability of matching distinguishable features between images taken during a temporal detection, which can be affected by imaging conditions. In some embodiments, the analyte can be fixed on a solid substrate or support. In some embodiments where there is or expects to be a spatial movement of an analyte during temporal detection, the location of the analyte with respect to a sample during each detection step can be determined and registered. Such spatial shift can then be corrected afterward during signal analysis using any art-recognized computer-implemented algorithms.

[0082] The length of time required to perform a temporal detection of the detection reagents (e.g., the length of time it takes to obtain a temporal sequence of signal signatures for the detection reagents) can vary, depending on the number of pre-determined subsequences to be detected or read and/or the number of available detection signals (e.g., fluorescent color and/or brightfield) to be read. In some embodiments, the length of time required to perform a temporal detection of the detection reagents can be, for example, but not limited to, 5 seconds, 10 seconds, 15 seconds, 20 seconds, 30 seconds, 1 mins, 2 mins, 3 mins, 4 mins, 5 mins, 15 mins, 30 mins, 1 hour, 2 hours, 4 hours, 6 hours, or longer.

[0083] The detection reagents can be detected by any means available in the art that is capable of detecting the specific signals on a given detection reagent generated during sequencing- or hybridization-based methods. Where the detection reagents (e.g., hybridized with decoder probes) are fluorescently labeled, suitable consideration of appropriate excitation sources can be readily determined. Possible sources can include but are not limited to arc lamp, xenon lamp, lasers, light emitting diodes or some combination thereof. The appropriate excitation source is used in conjunction with an appropriate optical detection system, for example an inverted fluorescent microscope, an epi-fluorescent microscope or a confocal microscope. Preferably, a microscope is used that can allow for detection with enough spatial resolution to separate distinct signals from individual detection reagents.

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[0084] Exemplary methods for detection of the detection reagents that are applicable to the methods described herein include, without limitations, the methods described in U.S. Pat. No. 7,473,767, US patent publication no. 2007/0166708, and US application number US 2010/0261026, all of which are incorporated by reference herein in its entirety.

[0085] Additional methods that can be used to detect optical signatures include, but are not limited to, any spectroscopic techniques, flow cytometry, or any art-recognized methods involving an optical scanner and/or a photodetector (e.g., without limitations, a charge-coupled devices, active pixel sensors, photodiode light sensors (e.g., LEDs), optical detectors, and any combinations thereof). Non-limiting examples of spectroscopic techniques can include absorption spectroscopy, emission spectroscopy, elastic scattering spectroscopy, reflection spectroscopy, impedance spectroscopy, inelastic spectroscopy, coherent or resonance spectroscopy, surface plasmon fluorescence spectroscopy, Raman spectroscopy, and any combinations thereof. Spectroscopy techniques can be used to detect light of any wavelengths, including, but not limited to, microwave, terahertz, infrared, near infrared, visible, ultraviolet, x-ray, gamma, and any combinations thereof.

[0086] Without wishing to be limited, in some embodiments, at least one pre-determined subsequence (e.g., individual bases or hybridization regions) can correspond to no optical signature; that is the absence of color can be considered as an additional color. In other embodiments, at least one pre-determined subsequence (e.g., individual bases or hybridization regions) can correspond to a compound optical signature, e.g., two or more simultaneous fluorescence in multiple channels during detection (e.g., by microscopy).

[0087] While a single area of a sample can interact with more than one probe reagents, the nucleic acid label can be designed such that any known or potential overlaps could be teased apart from the signal output. In the case where any probe may potentially overlap with all others, one can use the readout-by-hybridization variation and assign each probe a single unique hybridization sequence. Such approach can avoid multiple lengthy probe incubations and damaging stripping steps.

[0088] In some embodiments of the methods described herein, the signal signatures produced during any readout step (e.g., sequencing-based or hybridization-based) should be removed before advancing to the next pre-determined subsequence of the detection reagents. The removal of the signal signatures can be done by any methods known in the art, including, but not limited

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to, washing, heating, photo-bleaching, displacement, cleavage, enzymatic digestion, quenching, chemical degradation, bleaching, oxidation, and any combinations thereof.

[0089] In some embodiments, the decoder probes can be designed such that they can be simply washed out either with a plain buffer, or they can be modified by varying salt concentrations or using detergents or denaturants such as formamide or dimethyl sulfoxide (DMSO).

[0090] In other embodiments, the fluorescence or color signature of a readout step can be attenuated or eliminated by photo-bleaching the signal using sufficient optical exposure. In alternative embodiments, the fluorescence or color signature of a readout step can be attenuated or eliminated by subjecting the fluorescence or color signature to chemical degradation under appropriate conditions, e.g., using a reducing agent or oxidizing solution such as 0.01 M sodium periodate.

[0091] In some embodiments, the decoder probes can be displaced from their hybridization sites by introducing other reagents or probe displacers that have stronger binding affinities to those same sites. This can be done, for example, by using nucleic acid sequences that are longer than and/or have better complementarity than the decoder probe sequences. For example, to create "better complementarity" of the probe displacers, in some embodiments, mismatches can be seeded in the hybridization region. In other embodiments, the hybridization region can be preceded and/or post-ceded with a "toe-hold" of around 3-8 bases (e.g., 6 bases). In such embodiments, the "better complementarity" of the probe displacer can be outside of the hybridization region, which can make the design of the hybridization regions easier.

[0092] In some embodiments, enzymes can be used to displace, digest, cut and/or cleave the detectable labels, the decoder probe sequence, the hybridized complex (formed by the decoder probe and pre-determined subsequence), and/or the cleavable sequence linker to the hybridized complex, in order to remove the signal signatures. One example is to introduce a deoxyuridine into the decoder probe. This modified base can be cleaved using the enzyme mix known as USER, thereby cutting the decoder probe sequence into two parts. Since each part is now shorter, it is characterized by a lower melting temperature and can melt off the hybridization sites of the detection reagents. Alternatively, one of skill in the art can employ one of numerous art-recognized sequence-specific nucleases or restriction enzymes, which can cut either the decoder probe sequence, the pre-determined subsequence that has been hybridized with decoder probes,

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the cleavable sequence linker attached to the hybridized subsequence and/or the hybridized complex thereof, thereby removing the signal signature.

[0093] In some embodiments, thermal denaturing can be used to remove the signal signature from a previous readout. In some embodiments where sequencing is involved, thermal denaturing can be reduced or avoided by using a sequencing-by-ligation approach and by setting the nucleic acid label base that immediately follows the sequencing primer (in the direction of ligation) to an adenine. Correspondingly, the ligation probes should then include a (deoxy-)uracil in the primer-proximal position. Without wishing to be bound by theory, an enzyme such as USER, which cleaves DNA at uracils can be used to remove the ligation probe and ready the system for the next sequencing step. These fragments will have lower melting temperatures than their parent probes, and these temperatures can be designed to fall below the operating temperature (or require an acceptable denaturing temperature).

[0094] After detection of the pre-determined subsequences is completed in a temporally-sequential manner, in some embodiments, the method described herein can further comprise comparing the temporal order of the signal signatures with different identifiers of said at least one probe reagent, wherein an agreement between the temporal order of the signal signatures and a particular identifier of said at least one probe reagent identifies the analyte in the sample. In some embodiments, the method can further comprise measuring the intensity of the signal signatures generated from each subpopulation of the detection reagents. In some embodiments, the intensity of the signal signatures generated from each subpopulation of the detection reagents can indicate an amount of the analyte. 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 scheme of the detection reagents described herein. The comparing and intensity measuring steps can be performed, e.g., by a computer-implemented software or algorithm.

[0095] Types of signal signature(s) can vary upon different embodiments of detection reagents and/or decoder probes described herein. As used herein, the term "signal signature" refers to a change in, or occurrence of, a response or indicator that is detectable either by observation or instrumentally. In certain instances, the signal signature is fluorescence or a change in fluorescence, e.g., a change in fluorescence intensity, fluorescence excitation or emission wavelength distribution, fluorescence lifetime, and/or fluorescence polarization. By

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way of example only, the fluorescence can be produced by binding a fluorophore to a decoder probe, and/or by detecting the hybridization using a fluorescent dye, e.g., SYBR Gold, that lights up when nucleic acid sequence becomes double-stranded. In certain other instances, the signal signature can be radioactivity (i.e., radiation), including alpha particles, beta particles, nucleons, electrons, positrons, neutrinos, and gamma rays emitted by a radioactive substance such as a radionuclide. By way of example, the detection reagents and/or decoder probes can comprise an optical molecule or label, thus producing optical signatures. Examples of optical signatures can include, without limitations, signatures of fluorescent color, visible light, no-color, and any combinations thereof. In such embodiments, the optical signatures can be detected by optical imaging or spectroscopy.

Detection reagents (or detection molecules as used interchangeably herein)

[0096] Another aspect provided herein is a detection reagent, which can be, for example, used in the methods described herein for any multiplexing assays. The detection reagent comprises at least one probe reagent and at least one nucleic acid label, wherein said at least one nucleic acid label comprises at least one pre-determined subsequence to be detected in a temporally-sequential manner; wherein said at least one pre-determined subsequence forms an identifier of said at least one probe reagent; and wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together.

[0097] The detection reagents described herein can exist in different forms. By way of example only, in some embodiments, the detection reagent can be a detection molecule. In some embodiments, the detection reagent can be a detection particle. In some embodiments, the detection reagent can be multi-molecular.

[0098] As used herein, the term “conjugated" refers to two molecules being linked to each other, e.g., attaching a probe reagent to a nucleic acid label. The conjugation process can be performed, e.g., via a chemical reaction, or via a linker, which will be described later.

[0099] Depending on various applications and/or assay conditions (e.g., sensitivity, sample volume/concentration), a readout signal of a detection reagent can be amplified by increasing the number of the nucleic acid labels present in the detection reagent, e.g., by conjugating at least one probe reagent to a plurality of nucleic acid labels. In such embodiments, a plurality of the nucleic acid labels present in the detection reagent can range from about 2 to about 100,000,

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about 2 to about 10,000, about 2 to about 1,000, or about 2 to about 100. In some embodiments where the detection reagent comprises a particle as a hub, the number of possible nucleic acid labels present in the detection reagent can depend on the size of a particle. Generally, the larger the particle it is, the more nucleic acid labels can be incorporated into the detection reagent. For example, a particle of about 1-2 μm in size can allow incorporation of about 100,000 nucleic acid labels into the detection reagent. In some embodiments, there can be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 5000, 10000, 50000, 100000 nucleic acid labels present in the detection reagent. One of skill in the art can determine the optimum number of nucleic acid labels present the detection reagent without any undue experimentation.

[00100] The detection reagents described herein can be used in any biological assays for detection, identification and/or quantification of target molecules or analytes, including counting marked cells such as bacteria or cancer cells, in a sample. By way of example only, in some embodiments, the detection reagent can be adapted for use in immunofluorescence. In alternative embodiments, the detection reagent can be adapted for use in immunohistochemistry. In other embodiments, the detection reagent can be adapted for use in fluorescence in situ hybridization. In some embodiments, the detection reagent can be adapted for use in western blot. Depending on the nature of the sample and/or applications, the detection reagent can be adapted to be in any format, e.g., immobilized on a solid support, or in a solution or suspension phase. In certain embodiments, the detection reagent can be adapted to be present in a solution or suspension phase. The phrase "in a solution or suspension phase" as used herein generally refers to suspending the detection reagents in a liquid fluid, e.g., an aqueous buffer solution. Additional applications of the detection reagents and/or methods described herein will be discussed.

Probe reagents (or probe molecules as used interchangeably herein)

[00101] Each of the detection reagents described herein can comprise any number of probe reagents. In some embodiments, the detection reagent can comprise one or more probe reagents, e.g., at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10 or more probe reagents. In one embodiment, the detection reagent can comprise one probe reagent. In other embodiments, the detection reagent can comprise a plurality of probe reagents, e.g., ranging from about 2 to about 100,000 probe reagents, about 2 to about 10,000 probe reagents, about 2 to about 1,000 probe reagents, or about 2 to about 100 probe reagents. In

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some embodiments where the detection reagent comprises a particle as a hub, the number of possible probe reagents present in the detection reagent can depend on the size of a particle. Generally, the larger the particle it is, the more probe reagents can be incorporated into the detection reagent. For example, a particle of about 1-2 μm in size can allow incorporation of about 100,000 probe reagents into the detection reagent. In some embodiments, there can be about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 5000, 10000, 50000, 100000 probe reagents present in the detection reagent. One of skill in the art can determine the optimum number of probe reagents present the detection reagent without any undue experimentation.

[00102] As used interchangeably herein, the term "probe," "probe reagent" or "probe molecule" refers to an entity (e.g., but not limited to, a molecule, a particle, a composite entity, or a multi-molecular entity) that interacts with or binds to a target molecule or an analyte for the analysis of the target or the analyte. Typically the nature of the interaction or binding is noncovalent, e.g., by hydrogen, electrostatic, or van der Waals interactions, however, binding can also be covalent. Probe reagents can be entities (e.g., but not limited to, molecules, a particles, composite entities, or multi-molecular entities) capable of undergoing binding or molecular recognition events with target molecules. Probe reagents can be naturally-occurring, recombinant or synthetic. Examples of the probe reagent can include, but are not limited to, a nucleic acid, an antibody or a portion thereof, an antibody-like molecule, an enzyme, a cell, an antigen, a small molecule, a protein, a peptide, a peptidomimetic, an aptamer, and any combinations thereof. By way of example only, in immunohistochemistry, the probe reagent can include an antibody specific to the target antigen to be analyzed. An ordinary artisan can readily identify appropriate probe reagents for the target molecules or analytes of interest to be detected in various bioassays. In some embodiments, the probe reagent can be multi-molecular. For example, in one embodiment, the probe reagent can comprise a particle, an antibody, biotin and/or streptavidin, or any combinations thereof.

[00103] In some embodiments, the probe reagents can be modified by any means known to one of ordinary skill in the art. Methods to modify each type of probe reagents are well recognized in the art. Depending on the types of probe reagents, an exemplary modification includes, but is not limited to genetic modification, biotinylation, labeling (for detection purposes), chemical modification (e.g., to produce derivatives or fragments of the probe reagent),

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and any combinations thereof. In some embodiments, the probe reagent can be genetically modified. In some embodiments, the probe reagent can be biotinylated.

[00104] As used herein, the terms “proteins" and “peptides" are used interchangeably herein to designate a series of amino acid residues connected to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. The terms "protein", and "peptide", which are used interchangeably herein, refer to a polymer of protein amino acids, including modified amino acids (e.g., phosphorylated, glycated, etc.) and amino acid analogs, regardless of its size or function. Although "protein" is often used in reference to relatively large polypeptides, and "peptide" is often used in reference to small polypeptides, usage of these terms in the art overlaps and varies. The term "peptide" as used herein refers to peptides, polypeptides, proteins and fragments of proteins, unless otherwise noted. The terms "protein" and "peptide" are used interchangeably herein when referring to a gene product and fragments thereof. Thus, exemplary peptides or proteins include gene products, naturally occurring proteins, homologs, orthologs, paralogs, fragments and other equivalents, variants, fragments, and analogs of the foregoing.

[00105] As used herein, the term “peptidomimetic” refers to a molecule capable of folding into a defined three-dimensional structure similar to a natural peptide

[00106] The term “nucleic acids" used herein refers to polymers (polynucleotides) or oligomers (oligonucleotides) of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars and intersugar linkages. The term “nucleic acid” also includes polymers or oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly. Exemplary nucleic acids include, but are not limited to, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), locked nucleic acid (LNA), peptide nucleic acids (PNA), and polymers thereof in either single- or double-stranded form. Locked nucleic acid (LNA), often referred to as inaccessible RNA, is a modified RNA nucleotide. The ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' oxygen and 4' carbon. The bridge "locks" the ribose in the 3'-endo conformation. LNA nucleotides can be mixed with DNA or RNA residues in the oligonucleotide whenever desired. Such LNA oligomers are generally synthesized chemically. Peptide nucleic acid (PNA) is an artificially synthesized polymer similar to DNA or RNA. DNA and RNA have a deoxyribose and ribose sugar backbone, respectively, whereas PNA's backbone is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. PNA is generally synthesized chemically. Unless specifically limited, the term “nucleic

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acids" encompasses nucleic acids containing known analogs of natural nucleotides, which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer, et al., Nucleic Acid Res. 19:5081 (1991); Ohtsuka, et al., J. Biol. Chem. 260:2605-2608 (1985), and Rossolini, et al., Mol. Cell. Probes 8:91-98 (1994)). The term "nucleic acid" should also be understood to include, as equivalents, derivatives, variants and analogs of either RNA or DNA made from nucleotide analogs, and, single (sense or antisense) and double-stranded polynucleotides.

[00107] In some embodiments, the term "nucleic acid" described herein can include a modified nucleic acid. Modified nucleic acids are well known in the art. Thus, a nucleic acid described herein can comprise one or more nucleic acid modifications known in the art. For example, the nucleic acid can comprise one or more nucleic acid modifications selected from the group consisting of internucleotide linkage modifications (intersugar linkage modifications), sugar modifications, nucleobase modifications, backbone modifications/replacements, and any combinations thereof. Exemplary internucleotide linkage modifications include, but are not limited to, phosphorothioate, phosphorodithioate, phosphotriester (e.g. alkyl phosphotriester), aminoalkylphosphotriester, alkyl-phosphonate (e.g., methyl-phosphonate), selenophosphate, phosphoramidate (e.g., N-alkylphosphoramidate), boranophosphonate, and the like. Exemplary sugar modifications include, but are not limited to, 2′-*O*-Me (2′-*O*-methyl), 2′-*O*-MOE (2′-*O*-methoxyethyl), 2'-F, 2′-*O*-[2-(methylamino)-2-oxoethyl] (2′-*O*-NMA), 2'-*S*-methyl, 2'-O-CH_2-(4'-C) (LNA), 2'-O-CH_2CH_2-(4'-C) (ENA), 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), arabinose sugar, and the like. Exemplary nucleobase modifications include, but are not limited to, inosine, xanthine, hypoxanthine, nubularine, isoguanisine, tubercidine, 5-methylcytosine (5-me-C); 5-hydroxymethyl cytosine; xanthine; hypoxanthine; 2-aminoadenine; 6-methyl and other 6-alkyl derivatives of adenine and guanine; 2-propyl and other 2-alkyl derivatives of adenine and guanine; 2-thiouracil; 2-

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thiothymine; 2-thiocytosine; 5-propynyl uracil; 5-propynyl cytosine; 6-azouracil; 6-azocytosine; 6-azothymine; 5-uracil (pseudouracil); 4-thiouracil; 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines; 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines; 7-methyl and other 7-alkyl derivatives of adenine and guanine; 8-azaguanine; 8-azaadenine; 7-deazaguanine; 7-deazaadenine; 3-deazaguanine; 3-deazaadenin; universal base; and any combinations thereof. Exemplary backbone modifications include, but are not limited to, morpholino, cyclobutyl, pyrrolidine, peptide nucleic acid (PNA), aminoethylglycyl PNA (*aeg*PNA), backnone-extended pyrrolidine PNA (*bep*PNA), and the like.

[00108] The term "enzymes" as used here refers to a protein molecule that catalyzes chemical reactions of other substances without it being destroyed or substantially altered upon completion of the reactions. The term can include naturally occurring enzymes and bioengineered enzymes or mixtures thereof. Examples of enzyme families include kinases, dehydrogenases, oxidoreductases, GTPases, carboxyl transferases, acyl transferases, decarboxylases, transaminases, racemases, methyl transferases, formyl transferases, and α-ketodecarboxylases.

[00109] As used herein, the term "aptamers" means a single-stranded, partially single-stranded, partially double-stranded or double-stranded nucleotide sequence capable of specifically recognizing a selected non-oligonucleotide molecule or group of molecules. In some embodiments, the aptamer recognizes the non-oligonucleotide molecule or group of molecules by a mechanism other than Watson-Crick base pairing or triplex formation. Aptamers can include, without limitation, defined sequence segments and sequences comprising nucleotides, ribonucleotides, deoxyribonucleotides, nucleotide analogs, modified nucleotides and nucleotides comprising backbone modifications, branchpoints and nonnucleotide residues, groups or bridges. Methods for selecting aptamers for binding to a molecule are widely known in the art and easily accessible to one of ordinary skill in the art.

[00110] As used herein, the term "antibody" or "antibodies" refers to an intact immunoglobulin or to a monoclonal or polyclonal antigen-binding fragment with the Fc (crystallizable fragment) region or FcRn binding fragment of the Fc region. The term "antibodies" also includes "antibody-like molecules", such as fragments of the antibodies, e.g., antigen-binding fragments. Antigen-binding fragments can be produced by recombinant DNA techniques or by enzymatic or chemical cleavage of intact antibodies. "Antigen-binding

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fragments" include, inter alia, Fab, Fab', F(ab')2, Fv, dAb, and complementarity determining region (CDR) fragments, single-chain antibodies (scFv), single domain antibodies, chimeric antibodies, diabodies, and polypeptides that contain at least a portion of an immunoglobulin that is sufficient to confer specific antigen binding to the polypeptide. Linear antibodies are also included for the purposes described herein. The terms Fab, Fc, pFc', F(ab') 2 and Fv are employed with standard immunological meanings (Klein, Immunology (John Wiley, New York, N.Y., 1982); Clark, W. R. (1986) The Experimental Foundations of Modern Immunology (Wiley & Sons, Inc., New York); and Roitt, I. (1991) Essential Immunology, 7th Ed., (Blackwell Scientific Publications, Oxford)). Antibodies or antigen-binding fragments specific for various antigens are available commercially from vendors such as R&D Systems, BD Biosciences, e-Biosciences and Miltenyi, or can be raised against these cell-surface markers by methods known to those skilled in the art.

[00111] As used herein, the term "Complementarity Determining Regions" (CDRs; i.e. , CDR1, CDR2, and CDR3) refers to the amino acid residues of an antibody variable domain the presence of which are necessary for antigen binding. Each variable domain typically has three CDR regions identified as CDR1, CDR2 and CDR3. Each complementarity determining region may comprise amino acid residues from a "complementarity determining region" as defined by Kabat (i.e. about residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat et al. , Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (i.e. about residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk J. Mol. Biol. 196:901-917 (1987)). In some instances, a complementarity determining region can include amino acids from both a CDR region defined according to Kabat and a hypervariable loop.

[00112] The expression "linear antibodies" refers to the antibodies described in Zapata et al. , Protein Eng., 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (VH -CH1-VH-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

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[00113] The expression "single-chain Fv" or "scFv" antibody fragments, as used herein, is intended to mean antibody fragments that comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. (Plöckthun, The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer- Verlag, New York, pp. 269-315 (1994)).

[00114] The term "diabodies," as used herein, refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) Connected to a light-chain variable domain (VL) in the same polypeptide chain (VH - VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. (EP 404,097; WO 93/11161; Hollinger et ah, Proc. Natl. Acad. Sd. USA, P0:6444-6448 (1993)).

[00115] As used herein, the term “small molecules" refers to natural or synthetic molecules including, but not limited to, peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, aptamers, nucleotides, nucleotide analogs, organic or inorganic compounds (i.e., including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

[00116] The term “cells" used herein refers to any cell, prokaryotic or eukaryotic, including plant, yeast, worm, insect and mammalian. Mammalian cells include, without limitation; primate, human and a cell from any animal of interest, including without limitation; mouse, hamster, rabbit, dog, cat, domestic animals, such as equine, bovine, murine, ovine, canine, feline, etc. The cells may be a wide variety of tissue types without limitation such as; hematopoietic, neural, mesenchymal, cutaneous, mucosal, stromal, muscle spleen, reticuloendothelial, epithelial, endothelial, hepatic, kidney, gastrointestinal, pulmonary, T-cells etc. Stem cells, embryonic stem (ES) cells, ES- derived cells and stem cell progenitors are also included, including without

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limitation, hematopoeitic, neural, stromal, muscle, cardiovascular, hepatic, pulmonary, gastrointestinal stem cells, etc. Yeast cells may also be used as cells in some embodiments of the methods described herein. In some embodiments, the cells can be ex vivo or cultured cells, e.g. in vitro. For example, for ex vivo cells, cells can be obtained from a subject, where the subject is healthy and/or affected with a disease. Cells can be obtained, as a non-limiting example, by biopsy or other surgical means know to those skilled in the art.

[00117] As used herein, the term "antigens" refers to a molecule or a portion of a molecule capable of being bound by a selective binding agent, such as an antibody, and additionally capable of being used in an animal to elicit the production of antibodies capable of binding to an epitope of that antigen. An antigen may have one or more epitopes. The term "antigen" can also refer to a molecule capable of being bound by an antibody or a T cell receptor (TCR) if presented by MHC molecules. The term "antigen", as used herein, also encompasses T-cell epitopes. An antigen is additionally capable of being recognized by the immune system and/or being capable of inducing a humoral immune response and/or cellular immune response leading to the activation of B- and/or T-lymphocytes. This may, however, require that, at least in certain cases, the antigen contains or is linked to a Th cell epitope and is given in adjuvant. An antigen can have one or more epitopes (B- and T-epitopes). The specific reaction referred to above is meant to indicate that the antigen will preferably react, typically in a highly selective manner, with its corresponding antibody or TCR and not with the multitude of other antibodies or TCRs which may be evoked by other antigens. Antigens as used herein may also be mixtures of several individual antigens.

[00118] In some embodiments, the probe reagent can be an antibody or a portion thereof, or an antibody-like molecule. In such embodiments, the probe reagents can be used to, for example, detect and/or identify pathogen type or speices, the presence of cell or disease markers, cellular protein expression levels, phosphorylation or other post-translation modification state, or any combinations thereof. By way of example only, **Fig. 1** shows three different embodiments of the detection reagents comprising at least one (e.g., 1, 2, 3, 4, 5 or more) pathogen-specific antibodies (e.g., anti-E. Coli, anti-S. aureus, and anti-C. albicans).

[00119] In some embodiments, the probe reagent can be a nucleic acid (e.g., DNA, RNA, LNA, PNA, or any combinations thereof). In such embodiments, the nucleic acids can be used to determine, for example, the existence of characteristic cellular DNA or RNA sequences (such as

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in fluorescent in situ hybridization), RNA expression levels, miRNA presence and expression, and any combinations thereof, in various applications, e.g., for pathogen detection and/or identification.

[00120] In some embodiments, the probe reagent can be a protein or a peptide. In such embodiments, the protein or peptide can be essentially any proteins with known binding targets. Examples include, but are not limited to, innate-immune proteins (e.g., without limitations, MBL, Dectin-1, TLR2, and TLR4 and any proteins disclosed in U.S. Provisional Application No.: 61/508,957, the content of which is incorporated herein by reference in its entirety) and proteins comprising the chitin-binding domain. Such innate-immune proteins and chitin-binding domain proteins can be used to detect their corresponding pattern-recognition targets (e.g., microbes such as bacteria) and fungus, respectively. By way of example only, instead of using pathogen-specific antibodies as probe reagents in the detection reagents as shown in **Fig. 1**, innate-immune proteins (e.g., MBL) or chitin-binding domain proteins can be used as probe reagents for detection of pathogens. While such detection reagents can be used to detect pathogens, they may not be pathogen-specific, as compared to the ones using pathogen-specific antibodies as probes molecules.

[00121] In some embodiments, the probe reagent can be an aptamer. In some embodiments, the probe reagent can be a DNA or RNA aptamer. The aptamers can be used in various bioassays, e.g., in the same way as antibodies or nucleic acids described herein. By way of example only, **Fig. 2** shows some exemplary embodiments of the detection reagents comprising at least one (e.g., 1, 2, 3, 4, 5 or more) DNA aptamers (e.g., with a nucleotide sequence complementary to nuclear reprogramming factors, such as Oct4, Sox2, and Klf4). Such detection reagents can be used to determine RNA expression level of nuclear reprogramming factors in somatic cells for detecting, screening, or identifying stem cells (e.g., induced pluripotency stem cells).

[00122] In some embodiments, the probe reagent can be a cell surface receptor ligand. As used herein, a "cell surface receptor ligand" refers to a molecule that can bind to the outer surface of a cell. Exemplary, cell surface receptor ligand includes, for example, a cell surface receptor binding peptide, a cell surface receptor binding glycopeptide, a cell surface receptor binding protein, a cell surface receptor binding glycoprotein, a cell surface receptor binding organic compound, and a cell surface receptor binding drug. Additional cell surface receptor

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ligands include, but are not limited to, cytokines, growth factors, hormones, antibodies, and angiogenic factors.

[00123] When the detection reagents described herein are used as targeted delivery vehicles, e.g., for a diagnostic agent, in some embodiments, the probe reagent can be an endosomolytic ligand. As used herein, the term "endosomolytic ligand" refers to molecules having endosomolytic properties. Endosomolytic ligands can promote the lysis of and/or transport of the composition described herein, or its components, from the cellular compartments such as the endosome, lysosome, endoplasmic reticulum (ER), Golgi apparatus, microtubule, peroxisome, or other vesicular bodies within the cell, to the cytoplasm of the cell. Some exemplary endosomolytic ligands include, but are not limited to, imidazoles, poly or oligoimidazoles, linear or branched polyethyleneimines (PEIs), linear and branched polyamines, e.g. spermine, cationic linear and branched polyamines, polycarboxylates, polycations, masked oligo or poly cations or anions, acetals, polyacetals, ketals/polyketals, orthoesters, linear or branched polymers with masked or unmasked cationic or anionic charges, dendrimers with masked or unmasked cationic or anionic charges, polyanionic peptides, polyanionic peptidomimetics, pH-sensitive peptides, natural and synthetic fusogenic lipids, natural and synthetic cationic lipids.

[00124] In other embodiments, the probe reagent for use in delivery of an agent (e.g., a diagnostic agent) encapsulated within the detection reagents described herein can be a PK modulating ligand. As used herein, the terms "PK modulating ligand" and "PK modulator" refers to molecules which can modulate the pharmacokinetics of the composition described herein. Some exemplary PK modulator include, but are not limited to, lipophilic molecules, bile acids, sterols, phospholipid analogues, peptides, protein binding agents, vitamins, fatty acids, phenoxazine, aspirin, naproxen, ibuprofen, suprofen, ketoprofen, (S)-(+)-pranoprofen, carprofen, PEGs, biotin, and transthyretia-binding ligands (e.g., tetraiidothyroacetic acid, 2, 4, 6-triiodophenol and flufenamic acid).

[00125] In various embodiments, the detection reagent described herein can comprise one kind/species of probe reagents or different kinds/species of probe reagents. In some embodiments, the kind/species of the probe reagents present in the detection reagent can be the same. In other embodiments, the detection reagent can include at least one different kind/species of the probe reagents (e.g., 1, 2, 3, 4, 5, or 6 probe reagent species). In such embodiments, the

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distinct probe reagent species can be different from the others by types (e.g., antibodies vs. DNA aptamers), binding domains, and/or target analytes.

Nucleic acid labels

[00126] In accordance with embodiments of various aspects described herein, the nucleic acid label or nucleic acid tag comprises at least one pre-determined nucleic acid subsequence, which is used to identify an analyte or target. In some embodiments, the nucleic acid label or nucleic acid tag can comprise any number of the pre-determined nucleic acid subsequences, e.g., ranging from about 1 to about 100, from about 2 to about 80, or from about 3 to about 50. In some embodiments, the nucleic acid label or nucleic acid tag can comprise at least about 1, at least about 2, at least about 3, at least about 4, at least about 5, at least about 6, at least about 7, at least about 8, at least about 9, at least about 10, at least about 15, at least about 20, at least about 30, at least about 40, at least about 50, at least about 60, at least about 70 or more pre-determined nucleic acid subsequences. In some embodiments, the nucleic acid label or nucleic acid tag can comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70 or more pre-determined nucleic acid subsequences. Without wishing to be bound, the minimum number of pre-determined nucleic acid subsequences (n) required in the detection reagent can vary upon the number of distinct probes to be detected (X) and/or the number of distinct detectable labels (e.g., optical labels such as fluorescent labels or quantum dots) available to be used (Y), and n can be determined by the equation: $n = ceiling(\frac{\ln X}{\ln Y})$, where the mathematical function "ceiling" refers to rounding up a non-integer number to the nearest integer, when needed. For example, if 4 distinct detectable labels (Y) are used to distinguish 62 distinct probe reagents (X), n = ceiling (2.98) ~3. Therefore, at least three pre-determined nucleic acid subsequences are required in this example.

[00127] In some embodiments where the detectable labels include no-color (dark), the number of distinct detectable label available to be used can become Y+1. In such embodiments, n can be determined by the equation: $n = ceiling(\frac{\ln X}{\ln(Y+1)})$, and thus fewer pre-determined nucleic acid subsequences can be used. However, the combination in which all readout cycles are dark should not be allowed.

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[00128] Further, the pre-determined nucleic acid subsequences can be designed such that each readout cycle can light up in multiple colors. Thus, 2^Y instead of Y is used in the equation for n above and fewer pre-determined nucleic acid subsequences can thus be required. However, the combination in which all readout cycles are dark should not be allowed.

[00129] Additionally, while colors are generally used in a binary fashion, i.e., whether the color is present or not, in the above examples, the color intensity can also be used as a parameter of a signal signature. 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.

[00130] The pre-determined nucleic acid subsequences can be constructed from any types of nucleic acids, including, but not limited to, DNA, RNA, PNA, LNA and any combinations thereof.

[00131] Each of the pre-determined subsequences of the nucleic acid label can be independently of any length. In certain embodiments, the pre-determined subsequences can each independently comprise a length of about 1 nucleobase to about 100 nucleobases, from about 1 nucleobase to about 50 nucleobases, from about 2 nucleobases to about 50 nucleobases, from about 5 nucleobases to about 30 nucleobases, or from about 5 nucleobases to about 20 nucleobases. In some embodiments, the pre-determined subsequences can each independently comprise one or more nucleobases, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more nucleobases.

[00132] The achievable length of the pre-determined subsequences can affect the degree of multiplexibility. In some embodiments, the achievable length of the pre-determined subsequences can be a function of the decreasing fluorescence intensity over many cycles of stripped and rehybridization (i.e. diffusion of the sequenceable particles, degradation, increasing background noise). In the case of DNA or RNA based nucleic acid labels that are amplified in situ, modified base is incorporated during its synthesis (i.e., dUTP, biotin, Acrydite, aminoallele), enabling these detection reagents to be permanently embedded in a film (regardless of the film thickness) of functionalized polyacrylamide (i.e. Acrydite streptavidin, NHS ester Acrydite). The embedded material can then be stripped and rehybridized for many more cycles without altering its spatial architecture and minimizing the background noise.

[00133] Two or more pre-determined subsequences can be conjugated together within a nucleic acid label using any methods known in the art. In some embodiments, two or more pre-

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determined subsequences can be conjugated together by a sequence linker. The term "sequence linker" as used herein generally refers to an entity that connects two sequences or subsequences as described herein together.

[00134] In some embodiments, the sequence linker can be a direct bond or an atom such as nitrogen, oxygen or sulfur; a unit such as NR_1, C(O), C(O)NH, SO, SO_2, SO_2NH; or a chain of atoms. If needed, the two ends of the pre-determined subsequences can be linked together by providing on the two ends of the pre-determined subsequences complementary chemical functionalities that undergo a coupling reaction. In particular embodiments, the sequence linker is a direct bond, including, but not limited to, a phosphodiester bond. For example, a 3' carbon atom of a sugar base at the 5' end nucleotide of a first pre-determined subsequence can interact with the 5' carbon atom of another sugar base at the 3' end nucleotide of a second pre-determined subsequence to form a covalent bond, e.g., a phosphodiester bond. As such, two or more pre-determined subsequences can be bonded together to form a longer and contiguous pre-determined subsequence.

[00135] In some embodiments, the sequence linker can be a nucleotidic linker. The term "nucleotidic linker" as used herein refers to a linker of one nucleotide long or a sequence substantially comprising a plurality of nucleotides. In some embodiments, the nucleotidic linker can have a sequence length of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 30 or more nucleotides. The sequence length of the nucleotidic linker can vary with a number of factors, e.g., detection methods, and/or properties of optical labels. Without wishing to be bound by theory, in some embodiments, increasing the length of the nucleotidic linker can increase the flexibility of the nucleic acid label, e.g., to increase the binding frequency between a pre-determined sequence and a decoder probe, the term of which will be discussed later. However, too long a nucleotidic linker can result in a too long nucleic acid label, which could overlap with other nucleic acid labels of the detection reagents during an assay and thus reduce the quality and/or accuracy of the signal detection. One of skill in the art can determine the optimum length of the nucleotidic linker without undue experimentations.

[00136] The nucleotidic linker can be in any structure or conformation. In some embodiments, the nucleotidic linker can be in a structure selected from the group consisting of single-stranded, double-stranded, partially double-stranded, a hairpin, or any combinations thereof.

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[00137] In some embodiments, the sequence linker can be a bead or a nanoparticle acting as a hub. Accordingly, two or more pre-determined subsequences can be independently conjugated together via a bead or a nanoparticle.

[00138] Without wishing to be bound, while the sequence linker can be a direct bond, an atom, a nucleotidic linker, or any combinations thereof, the sequence linker can also include a sequence of amino acids, a polymer chain, a microbead, a nanobead, or any combinations thereof. To provide for the linkages between sequence linker, in some embodiments, different functionalities can be introduced to the ends of sequence linker and/or the pre-determined subsequences. Examples of functionalities include, but are not limited to, amide groups, including carbonic acid derivatives, ethers, esters, including organic and inorganic esters, amino, urethane, urea and any combinations thereof.

[00139] In some embodiments, the nucleic acid label is substantially a polynucleotide sequence containing one or more pre-determined subsequences. In such embodiments, any two pre-determined subsequences are either joined or conjugated together by a direct bond such as a phosphodiester bond (to produce longer contiguous subsequence), a nucleotidic linker of any desirable length, or any combinations thereof.

[00140] In such embodiments, the nucleic acid label of the detection reagent can be adapted to any configuration or structure. In some embodiments, the nucleic acid label can be single-stranded, double-stranded, partially double-stranded, a hairpin, linear, circular, branched, a concatemer, or any combinations thereof. In some embodiments, the nucleic acid label can be a linear polynucleotide sequence.

[00141] In some embodiments, the nucleic acid label can be a circular polynucleotide sequence. The advantage of using a circular nucleic acid label is that it can be amplified using rolling-circle amplification or hyperbranched rolling-circle amplification to generate a long continuous nucleic acid molecule that contains multiple copies of the same nucleic acid label sequences linked in series (also known as concatemer), thus resulting in an amplified signal. In some embodiments, instead of pre-forming the detection reagents comprising the circular nucleic acid label(s) and the probe reagent(s), detection reagents comprising linear polynucleotide(s) and the probe reagent(s) can be first synthesized. The circular nucleic acid labels can then added to hybridize with the linear polynucleotide(s) before or after the probe reagent(s) bind to the analytes. In such embodiments, while requiring an extra step, this approach can have the

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advantage over direct attachment of circular nucleic acid labels in that it does not require chemical modification of the nucleic acid label for conjugation with the probe reagents, thus resulting in a circular nucleic acid label that is compatible with a broader range of amplification enzymes. Furthermore, the linear polynucleotides can be smaller than the secondary circular nucleic acid labels, facilitating diffusion of the probe reagents (and the detection reagents) to their targets. In other embodiments, the linear polynucleotides can be circularized using a suitable double-stranded or single-stranded ligase, with or without the addition of a suitable ligation template. Without wishing to be bound by theory, such embodiments can avoid the extra hybridization that is required when a pre-circularized oligonucleotide is used instead.

[00142] When the detection reagents described herein are used as FISH probes, the nucleic acid labels and the FISH probes can be part of the same nucleic acid sequence construct, and thus the circularization can encompass the entire construct (e.g., both the nucleic acid labels and FISH probes). A schematic representation of exemplary FISH probes for SeqTagged FISH is shown in **Fig. 13**.

[00143] In some embodiments, the method described herein can be used to identify a class an analyte, e.g., a pathogen belongs to. For example, the method can be used to identify if a pathogen is a Gram-negative, Gram-positive, or some other class of pathogen, e.g., yeast. Thus, the method described herein can be used to as Gram test to determine whether a suspected pathogen is Gram positive, Gram negative or yeast. This can be useful for quickly identifying the type of infection in a subject and administering appropriate therapy. By way of example only, this can be accomplished using a detection reagent comprising a class-specific probe, also referred to as "Gram-stain like probe" herein. Again by way of example only, the probe can be a DNA probe for FISH.

[00144] In some embodiment, the FISH probe for identifying eubacteria can comprise the nucleotide sequence of SEQ ID NO: 4 (GCTGCCTCCCGTAGGAGT). An exemplary SeqTag labeled FISH-probe for identifying eubacteria can comprise the nucleotide sequence of SEQ ID NO: 5 (CTGCCTCCCGTAGGAGTTTTTTCGCTTTAGCCTAAGTGAAATC).

[00145] In some embodiments, the FISH probe for identifying yeast can comprise the nucleotide sequence of SEQ ID NO: 6 (CTCTGGCTTCACCCTATTC. An exemplary SeqTag labeled FISH-probe for identifying yeast can comprise the nucleotide sequence of SEQ ID NO: 7 (CTCTGGCTTCACCCTATTCTTTTTCGCTTTTTTGGGGAAAAGACA).

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[00146] In some embodiments, the FISH probe for identifying firmicutes can comprise the nucleotide sequence of SEQ ID NO: 8 (CGGAAGATTCCCTACTGC). An exemplary SeqTag labeled FISH-probe for identifying yeast can comprise the nucleotide sequence of SEQ ID NO: 9 (CGGAAGATTCCCTACTGCTTTTTCGCTTTCTGTAATGGAGTGGA).

[00147] In the SeqTag labeled FISH probes discussed above, each probe can be assigned a particular "signal signature" for each of the three readout steps. For example, colors can be labeled at B, C, and D. In some embodiments, each color can be from a different fluorophore, such as FAM, Cy3 and Cy5. Each signal signature can take advantage of multiple fluorophores simultaneously or even the same fluorophore multiple times, e.g., a SeqTag with signal signature corresponding to "DDDC."

[00148] An exemplary signal signature for identifying eubacteria, yeast or firmicutes is shown in **Table 1**. As shown, each class has a different assigned code for the first readout step. For example, for the first readout, eubacteria are assigned color C' yeast color D and firmicutes color B. On readout, eubacteria will show up as color C, yeast as color D, firmicutes as both colors B and C.

Table 1

Name	Assigned code				Effective code			
	S1	S2	S3		S1	S2	S3	
Eubacteria-Kempf	C				C			
All_yeast-Kempf	D				D			
Firmicutes-pB-00196	B				BC			

[00149] After identifying the class of pathogens, the pathogens can be further probed for identifying the specific pathogen or genus using the method described herein. For example, the detection reagent can comprise a pathogen specific probe that binds to a specific pathogen or genus of pathogens. For example, the probe can be a FISH probe that specifically binds to a specific pathogen. Some exemplary pathogen specific FISH probes are shown in **Table 2**.

Table 2

Name		probeBase FISH Sequence	Assigned code		Effective code	
SEQ ID NO:	Gram +					
10	Staph_spp-Kempf	TCCTCCATATCTCTGCGC	DD	B	BC DD	B
11	S_Aureus-Kempf	GAAGCAAGCTTCTCGTCCG	CD	B	BC CDDD	BB
12	Streptococcus_spp-	CACTCTCCCCTTCTGCAC	DD	D	BC DD	D

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	Kempf							
13	S_Pneumoniae-Kempf	GTGATGCAAGTGCACCTT	B	DC		BC	DDB	DDC
14	S_Pyogenes-Kempf	TTCCAAAGCGTACATTGGTT	C	DB		BC	DDC	DDB
15	S_Agalactiae-Kempf	GTAAACACCAAACMTCAGCG	D	DC		BC	DDD	DDC
16	B_subtilis-pB-00401	CGA AGG GGA CGT CCT ATC T	C	DD		BC	C	DD
17	L_acidophilus-pB-00711	CAG GCT TGC TCC TCG TTG	DD	C		BC	DD	C
	Gram -							
18	P_Aeruginosa-Kempf	TCTCGGCCTTGAAACCCC	B	B		C	B	B
19	K_Pneumoniae-Kempf	CCTACACACCAGCGTGCC	B	DD		C	B	DD
20	H_influenzae-pB-00348	CCG CAC TTT CAT CTT CCG	B	BC		C	B	BC
21	B_cepacia-pB-00346	CTG TGC GCC GGT TCT CTT	DD	B		C	DD	B
22	K_oxytoca-pB-01681	CTA CAA GAC TCC AGC CTG CC	DD	C		C	DD	C
23	E_coli-pB-02569-compl	ATG AGC AAA GGT ATT AAC TTT ACT CCC	B	C		C	B	C
24	Shewanella_spp-pB-01191-mod	AGC TAA TCC CAC CTA GGT TCA TC	BC	B		C	BC	B
25	H_pylori-pB-00361	CACACCTGACTGACTATCCCG	C	B		C	C	B
	Yeast							
26	C_Albicans-Kempf	GCCAAGGCTTATACTCGCT	C	BC		D	C	BC
27	C_Glabrata_Kempf	CCGCCAAGCCACAAGGACT	C	CD		D	C	CD
28	C_Krusei-Kempf	GATTCTCGGCCCCATGGG	BC	C		D	BC	C
29	C_Parapsilosis-Kempf	CCTGGTTCGCCAAAAAGGC	CD	C		D	CD	C

[00150] Exemplary SeqTag labeled FISH-probe for identifying a specific pathogen are shown in **Table 3**. As shown in **Table 3**, each specific pathogen can be assigned a specific signal signature based on an assigned color for each readout step and identity of a pathogen can be decoded using this table when the method is carried out using this set of assigned coding.

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Table 3

SEQ ID NO:	Name	SeqTag-labeled FISH-probe Sequence
	Gram +	
30	Staph_spp-Kempf	TCCTCCATATCTCTGCGCTTTTTCGCTTTCTGGAGAAAGGGCCATTTTTCGCTTTCGGTTCCAAAGACACTTTTTCGCTTTCTGGAGAAAGGGCCA
31	S_Aureus-Kempf	GAAGCAAGCTTCTCGTCCGTTTTTCGCTTTCTGGAGAAAGGGCCATTTTTCGCTTTCGGTTCCAAAGACACTTTTTCGCTTTGGAAGCACCTATTCC
32	Streptococcus_spp-Kempf	CACTCTCCCCTTCTGCACTTTTTCGCTTTCTGGAGAAAGGGCCATTTTTCGCTTTTCACGATCCCATGTATTTTTCGCTTTCTGGAGAAAGGGCCA
33	S_Pneumoniae-Kempf	GTGATGCAAGTGCACCTTTTTTTTCGCTTTGAAGCCGGTTATAGCTTTTTCGCTTTTAGGCATTAGCATTGTTTTTCGCTTTTCACGATCCCATGTA
34	S_Pyogenes-Kempf	TTCCAAAGCGTACATTGGTTTTTTTCGCTTTCGGTTCCAAAGACACTTTTTCGCTTTGGAAGCACCTATTCCTTTTTTCGCTTTTCACGATCCCATGTA
35	S_Agalactiae-Kempf	GTAAACACCAAACMTCAGCGTTTTTCGCTTTGAAGCCGGTTATAGCTTTTTTCGCTTTCTGGAGAAAGGGCCATTTTTCGCTTTTCACGATCCCATGTA
36	B_subtilis-pB-00401	CGA AGG GGA CGT CCT ATC TTTTTTCGCTTTTCACGATCCCATGTATTTTTCGCTTTGGAAGCACCTATTCCTTTTTCGCTTTTCACGATCCCATGTA
37	L_acidophilus-pB-00711	CAG GCT TGC TCC TCG TTGTTTTTCGCTTTCTGGAGAAAGGGCCATTTTTCGCTTTGAAGCCGGTTATAGCTTTTTCGCTTTCTGGAGAAAGGGCCA
	Gram -	
38	P_Aeruginosa-Kempf	TCTCGGCCTTGAAACCCCTTTTTCGCTTTTAGGCATTAGCATTGTTTTTCGCTTTCGGTTCCAAAGACAC
39	K_Pneumoniae-Kempf	CCTACACACCAGCGTGCCTTTTTCGCTTTTCACGATCCCATGTATTTTTCGCTTTTAGGCATTAGCATTGTTTTTCGCTTTTCACGATCCCATGTA
40	H_influenzae-pB-00348	CCG CAC TTT CAT CTT CCGTTTTTCGCTTTCGGTTCCAAAGACACTTTTTCGCTTTTAGGCATTAGCATTGTTTTTCGCTTTGAAGCCGGTTATAGC
41	B_cepacia-pB-00346	CTG TGC GCC GGT TCT CTTTTTTTCGCTTTCTGGAGAAAGGGCCATTTTTCGCTTTCGGTTCCAAAGACACTTTTTCGCTTTCTGGAGAAAGGGCCA
42	K_oxytoca-pB-01681	CTA CAA GAC TCC AGC CTG CCTTTTTCGCTTTCTGGAGAAAGGGCCATTTTTCGCTTTGAAGCCGGTTATAGCTTTTTCGCTTTCTGGAGAAAGGGCCA
43	E_coli-pB-02569-compl	ATG AGC AAA GGT ATT AAC TTT ACT CCCTTTTTCGCTTTTAGGCATTAGCATTGTTTTTCGCTTTGAAGCCGGTTATAGC
44	Shewanella_spp-pB-01191-mod	AGC TAA TCC CAC CTA GGT TCA TCTTTTTCGCTTTTAGGCATTAGCATTGTTTTTCGCTTTCGGTTCCAAAGACACTTTTTCGCTTTGGAAGCACCTATTCC

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45	H_pylori-pB-00361	CACACCTGACTGACTATCCCGTTTTTCGCTTTGGAAGCACCTATTCCTTTTTCGCTTTCGGTTCCAAAGACAC
	Yeast	
46	C_Albicans-Kempf	GCCAAGGCTTATACTCGCTTTTTTCGCTTTCGGTTCCAAAGACACTTTTTCGCTTTGGAAGCACCTATTCCTTTTTCGCTTTGAAGCCGGTTATAGC
47	C_Glabrata_Kempf	CCGCCAAGCCACAAGGACTTTTTTCGCTTTGAAGCCGGTTATAGCTTTTTCGCTTTGGAAGCACCTATTCCTTTTTCGCTTTTCACGATCCCATGTA
48	C_Krusei-Kempf	GATTCTCGGCCCCATGGGTTTTTCGCTTTTAGGCATTAGCATTGTTTTTCGCTTTGAAGCCGGTTATAGCTTTTTCGCTTTGGAAGCACCTATTCC
49	C_Parapsilosis-Kempf	CCTGGTTCGCCAAAAAGGCTTTTTCGCTTTCTGGAGAAAGGGCCATTTTTCGCTTTGAAGCCGGTTATAGCTTTTTCGCTTTGGAAGCACCTATTCC

[00151] As shown in **Table 3**, the "signal signature" can comprise multiple fluorescence "colors" in each step. For example, *E. coli* can be marked as red in step 1 and red+green in step 2 and blue in step 3. The "signal signature" can also be encoded in the brightness of the color at each step. For example, *E. coli* could be marked as red in step 1 and 3 times brighter red in step 2.

[00152] Exemplary hybridization sites for generating the different signal signatures at different steps using the exemplary SeqTag labeled FISH-probes described above are shown in **Table 4**.

Table 4

SEQ ID NO:	**Assigned "Color"**	
		Set1
50	B	CGCTTTCTGTAATGGAGTGGA
51	C	CGCTTTAGCCTAAGTGAAATC
52	D	CGCTTTTTTGGGGAAAAGACA
		Set 2
53	B	CGCTTTTAGGCATTAGCATTG
54	C	CGCTTTGGAAGCACCTATTCC
55	D	CGCTTTCTGGAGAAAGGGCCA
		Set 3
56	B	CGCTTTCGGTTCCAAAGACAC
57	C	CGCTTTGAAGCCGGTTATAGC
58	D	CGCTTTTCACGATCCCATGTA

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[00153] In some embodiments, multiple detection reagents can be used to identify a specific analyte. For example, a first set of probes can be used to identify whether the potential pathogen is gram-position, gram-negative and then another set of probes to identify it more specifically. Both probes would need to produce a detectable signal, allowing one to use, for example, **Table 3** to uniquely identify the pathogen.

[00154] In some embodiments, the nucleic acid label can be a concatemer, including a rolony or a DNA nanoball that is large enough to act as a particle rather than simply a strand of nucleic acid. Using a concatemer as a nucleic acid label can eliminate the need for in-situ enzymatic treatment of circular nucleic acid amplification, but it can also increase the overall molecular weight of the detection reagent and thus retard diffusion. Similar to the circular nucleic acid labels as described above, an exemplary approach of hybridizing concatemers with the linear polypeptides of the detection reagents after probe reagents bind to the analytes can be used to facilitate diffusion of the probe reagents (and the detection reagents) to their targets.

[00155] To increase the accuracy and/or specificity of the methods described herein, in various embodiments, the nucleic acid label can be designed for minimal cross-hybridization of bases with each other. Various art-recognized computational programs or algorithms are available to design nucleic acid sequences with minimal cross-hybridization. Thus, one of skill in the art can optimize the nucleic acid label sequence using any methods or algorithms known in the art.

[00156] In some embodiments, the nucleic acid labels described herein can be synthetic nucleic acid molecules (e.g., DNA, RNA, or DNA/RNA hybrids), and can be rationally-designed to have features that optimize labeling and detection of the detection reagents, and that prevent secondary structure formation. In some embodiments, a nucleic acid label is a designed polynucleotide sequence from about 50 to 50,000 bases long.

[00157] In some embodiments, the nucleic acid labels described herein can be designed to minimize predictable secondary structures, and/or be designed such that each nucleic acid label can hybridize only against its own target. In some embodiments, the nucleic acid labels described herein can be designed to be devoid of any secondary structure. Putative secondary structures (e.g. hairpins, folding, or internal base pairing) can be predicted by methods known in the art such as MFOLD. Without intending to be limited to any theory, in some embodiments, predictable secondary structure in the nucleic acid label can be minimized by avoiding inverted

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repeats and by skewing the backbone-specific content such that the backbone of the nucleic acid label is CT or GA-rich. Any art-recognized methods, e.g., MFOLD, can be used to verify if each nucleic acid label can hybridize only against its own target.

[00158] Sequences can also be screened to avoid common six-base-cutter restriction enzyme recognition sites. Selected sequences can be additionally subjected to predicted secondary structure analysis, and those with the least secondary structure may be chosen for further evaluation. Any program known in the art can be used to predict secondary structure, such as the MFOLD program (Zuker, 2003, Nucleic Acids Res. 31 (13):3406-15; Mathews et al., 1999, J. Mol. Biol. 288:911-940).

[00159] In some embodiments, the nucleic acid label can comprise only a subset of the A, G, C, T (and/or U) nucleotides or modified nucleotides thereof. In some embodiments, the nucleic acid label can comprise only one of the A, G, C, T (and/or U) nucleotides or modified nucleotides thereof. In some embodiments, the nucleic acid can comprise two of the A, C, T (and/or U) nucleotides or modified nucleotides thereof. In some embodiments, the nucleic acid label can comprise only three of the A, G, C, and T (and/or U) nucleotides or modified nucleotides thereof. In some embodiments, the nucleic acid label can comprise only four of the A, G, C, and T (and/or U) nucleotides or modified nucleotides thereof. The subset of the A, G, C and T (and/or U) nucleotides or modified nucleotide thereof can be selected from the group consisting of A; G; C; T; U; (A,G); (A,C); (G,T); (G,U); (C,T); (C,U); (G,T,U); (C,T,U); (A,C,G); (A,G,T); (A,G,U); (A,C,T); (A,C,U); (C,G,T); (C,G,U); (A,C,T,U); and (C,G,T,U).

[00160] Without wishing to be bound, the nucleic acid label can be a modified nucleic acid label. An exemplary modification of the nucleic acid label includes, without limitations, attaching one or more detectable molecules to the nucleic acid label (either to one end of or along the nucleic acid label sequence). The detectable molecule can be any optical molecule, including, but not limited to, a small-molecule dye, a fluorescent protein, a quantum dot, or any combinations thereof. In another embodiment, at least one end of the nucleic acid label can be modified to include a chemical functional group and/or a protein or peptide to facilitate the conjugation between the nucleic acid label and the probe reagent.

[00161] In some embodiments, at least one (including, e.g., at least two, at least three, at least four, at least five, at least six or more) nucleic acids or nucleotides present in the nucleic acid label can be modified. For example, the nucleic acid can comprise one or more nucleic acid

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modifications as described herein, e.g., selected from the group consisting of internucleotide linkage modifications (intersugar linkage modifications), sugar modifications, nucleobase modifications, backbone modifications/replacements, and any combinations thereof.

Conjugation between a nucleic acid label and a probe reagent

[00162] In accordance with embodiments of various aspects described herein, the detection reagent comprises at least one probe reagent and at least one nucleic acid label, wherein the probe reagent and the nucleic acid label can be conjugated together by any methods known in the art.

[00163] In some embodiments, the probe reagent and the nucleic acid label can be attached or conjugated together by a linker. As used herein, the term "linker" generally refers to an entity that connects the probe reagent and the nucleic acid label together. The linker can be monovalent or multivalent. The term "monovalent" as used herein refers to the capacity of a linker to join one probe reagent to one nucleic acid label. The term "multivalent" as used herein refers to the capacity of a linker to bind with one or more probe reagents and/or nucleic acid labels. In some embodiments, a multivalent linker can join at least one probe reagent to a plurality of nucleic acid labels (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10 or more nucleic acid labels).

[00164] In some embodiments, the term "linker" means an organic moiety that connects two parts of a compound. Such linkers typically comprise a direct bond or an atom such as oxygen or sulfur, a unit such as NH, C(O), C(O)NH, SO, SO_2, SO_2NH, SS, or a chain of atoms, such as substituted or unsubstituted C_1-C_6 alkyl, substituted or unsubstituted C_2-C_6 alkenyl, substituted or unsubstituted C_2-C_6 alkynyl, substituted or unsubstituted C_6-C_{12} aryl, substituted or unsubstituted C_5-C_{12} heteroaryl, substituted or unsubstituted C_5-C_{12} heterocyclyl, substituted or unsubstituted C_3-C_{12} cycloalkyl, where one or more methylenes can be interrupted or terminated by O, S, S(O), SO_2, NH, C(O).

[00165] In some embodiments, the linker is a branched linker. The branchpoint of the branched linker may be at least trivalent, but can be a tetravalent, pentavalent or hexavalent atom, or a group presenting such multiple valencies. In some embodiments, the branchpoint is -N, -N(R)-C, -O-C, -S-C, -SS-C, -C(O)N(R)-C, -OC(O)N(R)-C, -N(R)C(O)-C, or -N(R)C(O)O-C; wherein R is independently for each occurrence H or optionally substituted alkyl. In some embodiments, the branchpoint is glycerol or derivative thereof.

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[00166] In some embodiments, linker comprises a cleavable linking group. As used herein, a "cleavable linking group" is a chemical moiety which is sufficiently stable outside the cell, but which upon entry into a target cell is cleaved to release the two parts the linker is holding together. In a preferred embodiment, the cleavable linking group is cleaved at least 10 times or more, preferably at least 100 times faster in the target cell or under a first reference condition (which can, e.g., be selected to mimic or represent intracellular conditions) than in the blood or serum of a subject, or under a second reference condition (which can, e.g., be selected to mimic or represent conditions found in the blood or serum).

[00167] Cleavable linking groups are susceptible to cleavage agents, e.g., pH, redox potential or the presence of degradative molecules. Generally, cleavage agents are more prevalent or found at higher levels or activities inside cells than in serum or blood. Examples of such degradative agents include: redox agents which are selected for particular substrates or which have no substrate specificity, including, e.g., oxidative or reductive enzymes or reductive agents such as mercaptans, present in cells, that can degrade a redox cleavable linking group by reduction; esterases; amidases; endosomes or agents that can create an acidic environment, e.g., those that result in a pH of five or lower; enzymes that can hydrolyze or degrade an acid cleavable linking group by acting as a general acid, peptidases (which can be substrate specific) and proteases, and phosphatases.

[00168] A linker can include a cleavable linking group that is cleavable by a particular enzyme. The type of cleavable linking group incorporated into a linker can depend on the cell to be targeted. For example, for liver targeting, cleavable linking groups can include an ester group. Liver cells are rich in esterases, and therefore the linker will be cleaved more efficiently in liver cells than in cell types that are not esterase-rich. Other cell-types rich in esterases include cells of the lung, renal cortex, and testis.

[00169] Linkers that contain peptide bonds can be used when targeting cell types rich in peptidases, such as liver cells and synoviocytes.

[00170] In some embodiments, cleavable linking group is cleaved at least 1.25, 1.5, 1.75, 2, 3, 4, 5, 10, 25, 50, or 100 times faster in the cell (or under in vitro conditions selected to mimic intracellular conditions) as compared to blood or serum (or under in vitro conditions selected to mimic extracellular conditions). In some embodiments, the cleavable linking group is cleaved by less than 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, or 1% in the blood (or in

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vitro conditions selected to mimic extracellular conditions) as compared to in the cell (or under in vitro conditions selected to mimic intracellular conditions).

[00171] Exemplary cleavable linking groups include, but are not limited to, redox cleavable linking groups (e.g., -S-S- and $-C(R)_2$-S-S-, wherein R is H or C_1-C_6 alkyl and at least one R is C_1-C_6 alkyl such as CH_3 or CH_2CH_3); phosphate-based cleavable linking groups (e.g., -O-P(O)(OR)-O-, -O-P(S)(OR)-O-, -O-P(S)(SR)-O-, -S-P(O)(OR)-O-, -O-P(O)(OR)-S-, -S-P(O)(OR)-S-, -O-P(S)(ORk)-S-, -S-P(S)(OR)-O-, -O-P(O)(R)-O-, -O-P(S)(R)-O-, -S-P(O)(R)-O-, -S-P(S)(R)-O-, -S-P(O)(R)-S-, -O-P(S)(R)-S-, . -O-P(O)(OH)-O-, -O-P(S)(OH)-O-, -O-P(S)(SH)-O-, -S-P(O)(OH)-O-, -O-P(O)(OH)-S-, -S-P(O)(OH)-S-, -O-P(S)(OH)-S-, -S-P(S)(OH)-O-, -O-P(O)(H)-O-, -O-P(S)(H)-O-, -S-P(O)(H)-O-, -S-P(S)(H)-O-, -S-P(O)(H)-S-, and -O-P(S)(H)-S-, wherein R is optionally substituted linear or branched C_1-C_{10} alkyl); acid celavable linking groups (e.g., hydrazones, esters, and esters of amino acids, -C=NN- and -OC(O)-); ester-based cleavable linking groups (e.g., -C(O)O-); peptide-based cleavable linking groups, (e.g., linking groups that are cleaved by enzymes such as peptidases and proteases in cells, e.g., $-NHCHR^AC(O)NHCHR^BC(O)$-, where R^A and R^B are the R groups of the two adjacent amino acids). A peptide based cleavable linking group comprises two or more amino acids. In some embodiments, the peptide-based cleavage linkage comprises the amino acid sequence that is the substrate for a peptidase or a protease found in cells.

[00172] In some embodiments, an acid cleavable linking group is cleaveable in an acidic environment with a pH of about 6.5 or lower (e.g., about 6.0, 5.5, 5.0, or lower), or by agents such as enzymes that can act as a general acid.

[00173] In addition to covalent linkages, two parts of a compound can be linked together by an affinity binding pair. The term "affinity binding pair" or "binding pair" refers to first and second molecules that specifically bind to each other. One member of the binding pair is conjugated with first part to be linked while the second member is conjugated with the second part to be linked. As used herein, the term "specific binding" refers to binding of the first member of the binding pair to the second member of the binding pair with greater affinity and specificity than to other molecules.

[00174] Exemplary binding pairs include any haptenic or antigenic compound in combination with a corresponding antibody or binding portion or fragment thereof (e.g., digoxigenin and anti-digoxigenin; mouse immunoglobulin and goat antimouse immunoglobulin) and

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nonimmunological binding pairs (e.g., biotin-avidin, biotin-streptavidin, biotin-neutravidin, hormone [e.g., thyroxine and cortisol-hormone binding protein, receptor-receptor agonist, receptor-receptor antagonist (e.g., acetylcholine receptor-acetylcholine or an analog thereof), IgG-protein A, IgG-protein G, IgG-synthesized protein AG, lectin-carbohydrate, enzyme-enzyme cofactor, enzyme-enzyme inhibitor, and complementary oligonucleotide pairs capable of forming nucleic acid duplexes), and the like. The binding pair can also include a first molecule which is negatively charged and a second molecule which is positively charged.

[00175] One example of using binding pair conjugation is the biotin-avidin, biotin-streptavidin or biotin-neutravidin conjugation. In this approach, one of the molecule or the peptide is biotinylated and the other is conjugated with avidin or streptavidin. Many commercial kits are also available for biotinylating molecules, such as proteins.

[00176] Another example of using binding pair conjugation is the biotin-sandwich method. *See, e.g.*, example Davis et al., Proc. Natl. Acad. Sci. USA, 103: 8155-60 (2006). The two molecules to be conjugated together are biotinylated and then conjugated together using at least one tetravalent avidin-like molecule (e.g., avidin, streptavidin, or neutravidin) as a linker.

[00177] Accordingly, in some embodiments, both the nucleic acid label(s) and probe reagent(s) can be biotinylated and then linked together using an avidin-like molecule (e.g., avidin, streptavidin, or neutravidin). In one embodiment, neutravidin and/or streptavidin is used as a linker to bridge together the biotinylated nucleic acid label(s), e.g., DNA sequence(s), and biotinylated probe reagent(s), e.g., antibody. Without wishing to be bound by theory, each avidin-like molecule (e.g., avidin, streptavidin, or neutravidin) generally has four binding sites, so at most four molecules can be linked together. For example, one biotinylated probe reagent can be linked, via an avidin-like molecule (e.g., avidin, streptavidin or neutravidin), to three biotinylated nucleic acid labels, or in any other combinations (e.g., two biotinylated probe reagents linked to two biotinylated nucleic acid labels).

[00178] Biotinylation of a nucleic acid label (i.e., attaching a biotin to a nucleic acid label) can occur at any location of the nucleic acid label. In some embodiments, the nucleic acid label can by synthesized or modified with a terminal biotin, i.e., the nucleic acid label can have a biotin at its 5' end and/or 3' end. In other embodiments, the nucleic acid label can by synthesized or modified with an internal biotin, i.e., the nucleic acid label can have a biotin anywhere between its 5' and 3' ends, but not at its 5' and/or 3' ends. Such internal biotinylation can leave at least

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one end (e.g., both ends) of the nucleic acid label accessible, allowing the nucleic acid label to be circularized after the nucleic acid label has bound, which can in turn, for example, enable rolling circle amplification as described earlier. Chemical modification of the nucleic acid label, e.g., to attach a terminal and/or an internal biotin to the nucleic acid label after synthesis, is within one of skill in the art.

[00179] For some embodiments where the probe reagent is a nucleic acid, an example of using binding pair conjugation is double-stranded nucleic acid conjugation. In this approach, the first part to be linked is conjugated is with linked a first strand first strand of the double-stranded nucleic acid and the second part to be linked is conjugated with the second strand of the double-stranded nucleic acid. Nucleic acids can include, without limitation, defined sequence segments and sequences comprising nucleotides, ribonucleotides, deoxyribonucleotides, nucleotide analogs, modified nucleotides and nucleotides comprising backbone modifications, branchpoints and nonnucleotide residues, groups or bridges.

[00180] In some embodiments, the linker can be a linker molecule. Examples of linker molecules can include, but are not limited to, a polymer, sugar, nucleic acid, peptide, protein, hydrocarbon, lipid, polyethelyne glycol, crosslinker, or combination thereof.

[00181] Non-limiting examples of crosslinkers that can be used as linker molecules can include, but are not limited to, amine-to-amine crosslinkers (e.g., but are not limited to the ones based on NHS-ester and/or imidoester reactive groups), amine-to-sulfhydryl crosslinkers, carboxyl-to-amine crosslinkers (e.g., but are not limited to, carbodiimide crosslinking agents such as DCC, and/or EDC (EDAC); and/or N-hydroxysuccinimide (NHS)), photoreactive crosslinkers (e.g., but not limited to, aryl azide, diazirine and any art-recognized photo-reactive (light-activated) chemical crosslinking reagents), sulfhydryl-to-carbohydrate crosslinkers (e.g., but are not limited to the ones based on malemide and/or hydrazide reactive groups), sulfhydryl-to hydroxyl crosslinkers (e.g., but are not limited to the ones based on maleimide and/or isocyanate reactive groups), sulfhydryl-to-sulfhydryl crosslinkers (e.g., but are not limited to, maleimide and/or pyridyldithiol reactive groups), sulfo-SMCC crosslinkers, sulfo-SBED biotin label transfer reagents, sulfhydryl-based biotin label transfer reagents, photoreactive amino acids (e.g., but are not limited to diazirine analogs of leucine and/or methionine), NHS-azide Staudinger ligation reagents (e.g., but are not limited to, activated azido compounds), NHS-phosphine Staudinger ligation reagents (e.g., but are not limited to, activated phosphine

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compounds), and any combinations thereof.

[00182] In some embodiments, any commercially available crosslinkers (e.g., but not limited to the ones from Thermo Scientific or Piercenet, Rockford, IL) can be used as a linker molecule herein.

[00183] In some embodiments, the term "linker" as used herein can be a physical substrate. In some embodiments, the physical substrate is a particle. The particle can be of any shape, e.g., spheres; rods; shells; beads, tubes, wires, and prisms; and these particles can be part of a network.

[00184] The particles can be made of any materials. In some embodiments, the particle can comprise a material selected from the group consisting of metal (e.g., gold, or iron), metal oxides (e.g., iron oxide), plastic, glass, polymer (e.g., polystyrene), and any combinations thereof.

[00185] For in vivo purposes, e.g., disease and/or pathogen diagnosis and/or targeted drug delivery, the polymer can be biocompatible and/or biodegradable. The term "biocompatible polymer" refers to polymers which, in the amounts employed, are non-toxic and substantially non-immunogenic when used internally in the patient and which are substantially insoluble in the body fluid of the mammal. Examples of non-biodegradable biocompatible polymers include, by way of example, cellulose acetates (including cellulose diacetate), ethylene vinyl alcohol copolymers, hydrogels (e.g., acrylics), polyacrylonitrile, polyvinylacetate, cellulose acetate butyrate, nitrocellulose, copolymers of urethane/carbonate, copolymers of styrene/maleic acid, and any combinations thereof. Biodegradable polymers are known in the art, e.g., without limitations, linear-chain polymers such as polylactides, polyglycolides, polycaprolactones, polyanhydrides, polyamides, polyurethanes, polyesteramides, polyorthoesters, polydioxanones, polyacetals, polyketals, polycarbonates, polyorthocarbonates, polyphosphazenes, polyhydroxybutyrates, polyhydroxyvalerates, polyalkylene oxalates, polyalkylene succinates, poly(malic acid), poly(amino acids), polyvinylpyrrolidone, polyethylene glycol, polyhydroxycellulose, chitin, chitosa, and copolymers, terpolymers and any combinations thereof. Other biodegradable polymers include, for example, fibrin, gelatin, collagen, and any combinations thereof.

[00186] The particles can be of any size for the purpose of various aspects described herein, provided that the particle size does not significantly affect the diffusion property of the detection reagent. For example, the particle size can range from 5 nm to 1 mm, from about 10 nm to about

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500 μm, or from about 50 nm to about 250 μm. In some embodiments, a particle described herein is a nanoparticle or a microparticle. As used herein, the term "nanoparticle" refers to particles that are on the order of 10^{-9} or one billionth of a meter and below 10^{-6} or 1 millionth of a meter in size. The term "microparticle" as used herein refers to particles that are on the order of 10^{-6} or one millionth of a meter and below 10^{-3} or 1 thousandth of a meter in size. In some embodiments, the particle can be selected from a group consisting of a gold nanoparticle or microparticle, a paramagnetic nanoparticle or microparticle, a magnetic nanoparticle or microparticle, a polystyrene nanoparticle or microparticle, a nanotube or a microtube, a nanowire or a microwire, and any combinations thereof.

[00187] The particles can be adapted to possess at least one additional property, depending on various applications. In some embodiments, the particles can be adapted to be magnetic responsive or paramagnetic-responsive, e.g., magnetic or paramagnetic particles. In some embodiments, the particles can be adapted to be a delivery vehicle. For example, in some embodiments, the particles can be encapsulated with a therapeutic agent, e.g., for targeted drug delivery to treat a disease or disorder.

[00188] In some embodiments, the particles can be modified. For example, the particles can be conjugated with proteins, peptides, nucleic acids, or any combinations thereof. In one embodiment, the particles can be surface-conjugated or coated with one member of the binding pair as described above (e.g., for biotin-streptavidin interaction, streptavidin-coated particles can be used for the purpose of various aspects described herein). In some embodiments, the particles can be surface-activated with functional groups (e.g., but not limited to, amine, carboxylic acid, epoxy, tosyl, silane, and any combinations thereof), e.g., to provide binding sites for probe reagents and/or nucleic acid labels. Methods for surface modifications of particles, such as nanoparticles or microparticles, are well known in the art. One of skill in the art can readily modify or activate the surface of particles using any art-recognized reactions, e.g., covalently through crosslinkers, or through protein interaction.

[00189] The particles described herein can be used in conjunction with the organic linker described above, to form a conjugate linker, or the particles can be used alone as a linker. Both the nucleic acid labels and the probe reagents can be coupled to the particles using a multitude of methods. These include, but are not limited to, direct covalent attachment such as using chemical crosslinkers based on chemistries such as NHS, maleimide, tosyl, isocyanide, etc.; chemical

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linkage such as using EDC chemistry, thiol adsorption to gold, vinyl/acrylate radical reaction, acrylate-based addition (of e.g., thiols); and protein mediated couplings based on proteins such as streptavidin (and its relatives such as avidin, neutravidin, etc.), protein A, and secondary antibodies, and any combinations thereof.

[00190] The particles can act as hubs that facilitate a conjugation of multiple nucleic acid labels to single or multiple probes. Depending on applications and/or properties of nucleic acid labels and/or probe reagents, particles can be used as hubs for multi-conjugation. For example, in some embodiments, by acting as a hub, the particles can allow multiple nucleic acid labels to be present at each location (e.g., location of a target molecule or analyte) where the probe binds. Accordingly, the signal generated from the detection reagent can be amplified and thus greater than if only a single label was present. This is especially desirable where antigens/targets or analytes are sparse in a sample.

[00191] In some embodiments, the particles can allow multiple probes to be arranged in proximity to each other. Thus, the particles can allow several weaker binding events to combine into strong binding. This "avidity action" can transform probe reagents with individually weak affinities into effective sensors.

[00192] In some embodiments, by acting as a hub, the particles can allow each probe reagent to conjugate to multiple nucleic acid labels. This capability can eliminate the need for other amplification methods (e.g., rolling circle amplification) as multiple nucleic acid labels corresponding to a probe reagent can be used as a form of signal amplification. In some embodiments, this capability can also be used to separate the detection reagents with one nucleic acid label from the ones with different nucleic acid labels, which can, in turn, enable, for example, sequencing by single-base extension. Furthermore, different nucleic acid labels can be conjugated to the particles at controlled ratios, and the ratios can encode additional information. By way of example only, to capture in situ information of one or more enzymes' behavior (e.g., relative enzyme concentration) in the presence of an analyte, two or more different nucleic acid labels can be conjugated to the particles, wherein a subpopulation of the nucleic acid labels corresponding to the probe reagent is not cleavable, while other subpopulations of the nucleic acid labels are each adapted to be cleavable in the presence of their respective enzymes. In such embodiments, the cleavable nucleic acid labels can be conjugated to the particles by cleavable peptide bonds specific for corresponding enzymes. By comparing the ratios of signals generated

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from the various nucleic acid labels, one would be able to determine the relative enzyme concentrations in the presence of an analyte.

[00193] The arrangement of the probe reagent(s) and/or nucleic acid label(s) on the particles can vary with a number of factors, e.g., applications, properties of probe reagents and/or nucleic acid labels, sample properties, and/or analytes of interests. In some embodiments, the probe reagents and nucleic acid labels can be conjugated to the particle directly (see, e.g., **Fig. 4A**). In some embodiments, one component can be linked to the particle through the other. In such embodiments, the probe reagents can be conjugated or linked to the particle through the nucleic acid labels (see, e.g., **Fig. 4B**), e.g., to allow the probe reagents being more accessible by the corresponding analytes in a sample.

[00194] In some embodiments, the detection reagents can further comprise at least one substrate linker conjugated to the particle. In such embodiment, the substrate linker can allow the detection reagents to be immobilized to a solid support or substrate.

Detectable molecules or detectable labels

[00195] A detectable molecule or detectable label can be covalently attached to a decoder probe or complementary nucleobase before or after the decoder probe or the complementary nucleobase is attached to the pre-determined subsequences or hybridization sites of a nucleic acid label. For example, in attaching a detectable label to a decoder probe, the label can be covalently attached by incorporation of a nucleotide containing a detectable label into the nucleic acid during its synthesis, but before it is hybridized the pre-determined subsequence of the nucleic acid label. Alternatively, during the synthesis of a decoder probe sequence, a nucleotide containing a detectable label acceptor group can be included, and the detectable label can be added to the decoder probe after its synthesis, either before or after it is hybridized to the pre-determined subsequences of the nucleic acid label. Alternatively, the detectable label can be indirectly attached to the decoder probe, for example, by incorporating a nucleotide containing a ligand-binding molecule (e.g., biotin) into the decoder probe during synthesis, and by adding a ligand (e.g., streptavidin) that is covalently attached to the detectable molecule, or vice versa.

[00196] In some embodiments, the ratios of a detectable label to a decoder probe can range from about 1:1 to about 100:1, from 1:1 to about 50:1, from about 1:1 to about 25:1, from about

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1:1 to about 10:1, or from about 1:1 to about 5:1. When a decoder probe comprises more than one detectable label, each detectable label can be attached to a nucleotide of the decoder probe.

[00197] A detectable label or a detectable molecule can be attached to any nucleotide including both natural and non-natural nucleotides. A nucleotide contains three parts, a phosphate group, a pentose five-carbon sugar molecule, and an organic base. In RNA, the pentose is ribose and in DNA it is deoxyribose and so nucleotides for incorporation into RNA are called ribonucleotides and nucleotides for incorporation into DNA are called deoxyribonucleotides. Three bases adenine, guanine, and cytosine are found in both DNA and RNA while thymine is normally found only in DNA and uracil is normally found only in RNA. Nucleotides can have one, two or three attached phosphate groups and are sometimes referred to as nucleoside phosphates. Nucleotides can contain modified nucleosides having modified bases (e.g., 5-methyl cytosine) and modified sugar groups (e.g., 2′O-methyl ribosyl, 2′O-methoxyethyl ribosyl, 2′fluoro ribosyl, 2′amino ribosyl, and the like). An example of non-natural bases that are used in the art are isocytidine and isoguanine.

[00198] A detectable label or a detectable molecule as used herein is intended to mean an individual measurable moiety, such as a radioisotope, fluorochrome, dye, enzyme (including its effect on a substrate), nanoparticle, chemiluminescent marker, biotin, or other moiety known in the art that is measurable by analytical methods. A detectable label or a detectable molecule can be attached to a nucleotide using methods well known in the art and exemplified herein.

[00199] Without limitations, examples of a detectable label or a detectable molecule that can be utilized by some aspects described herein can include optical reporters or optical labels. Suitable optical reporters or optical labels include, but are not limited to, fluorescent reporters and chemiluminescent groups. A wide variety of fluorescent reporter dyes are known in the art. Typically, the fluorophore is an aromatic or heteroaromatic compound and can be a pyrene, anthracene, naphthalene, acridine, stilbene, indole, benzindole, oxazole, thiazole, benzothiazole, cyanine, carbocyanine, salicylate, anthranilate, coumarin, fluorescein, rhodamine or other like compound. Suitable fluorescent reporters include xanthene dyes, such as fluorescein or rhodamine dyes, including, but not limited to, Alexa Fluor® dyes (InvitrogenCorp.; Carlsbad, Calif), fluorescein, fluorescein isothiocyanate (FITC), Oregon GreenTM, rhodamine, Texas red, tetrarhodamine isothiocynate (TRITC), 5-carboxyfluorescein (FAM), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), tetrachlorofluorescein (TET), 6-carboxyrhodamine (R6G),

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N,N,N,N'-tetramefhyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX). Suitable fluorescent reporters also include the naphthylamine dyes that have an amino group in the alpha or beta position. For example, naphthylamino compounds include l-dimethylamino-naphthyl-5-sulfonate, l-anilino-8-naphthalene sulfonate, 2-p-toluidinyl-6-naphthalene sulfonate, and 5-(2'-aminoethyl)aminonaphthalene-l-sulfonic acid (EDANS). Other fluorescent reporter dyes include coumarins, such as 3-phenyl-7-isocyanatocoumarin; acridines, such as 9-isothiocyanatoacridine and acridine orange; N-(p(2-benzoxazolyl)phenyl)maleimide; cyanines, such as Cy2, indodicarbocyanine 3 (Cy3), indodicarbocyanine 5 (Cy5), indodicarbocyanine 5.5 (Cy5.5), 3-(-carboxy-pentyl)-3'ethyl-5,5'-dimethyloxacarbocyanine (CyA); 1H,5H,11H, 15H-Xantheno[2,3,4-ij:5,6,7-i'j']diquinolizin-18-ium, 9-[2(or 4)-[[[6-[2,5-dioxo-l-pyrrolidinyl)oxy]-6-oxohexyl] amino]sulfonyl]-4(or 2)-sulfophenyl]-2,3,6,7,12,13,16,17octahydro-inner salt (TR or Texas Red); BODIPYTM dyes; benzoxadiazoles; stilbenes; pyrenes; and the like. Many suitable forms of these fluorescent compounds are available and can be used.

[00200] Examples of fluorescent proteins suitable for use as imaging agents include, but are not limited to, green fluorescent protein, red fluorescent protein (e.g., DsRed), yellow fluorescent protein, cyan fluorescent protein, blue fluorescent protein, and variants thereof (see, e.g., U.S. Pat. Nos. 6,403, 374, 6,800,733, and 7,157,566). Specific examples of GFP variants include, but are not limited to, enhanced GFP (EGFP), destabilized EGFP, the GFP variants described in Doan et al, *Mol. Microbiol,* 55:1767-1781 (2005), the GFP variant described in Crameri et al, *Nat. Biotechnol.,* 14:315319 (1996), the cerulean fluorescent proteins described in Rizzo et al, *Nat. Biotechnol,* 22:445 (2004) and Tsien, *Annu. Rev. Biochem.,* 67:509 (1998), and the yellow fluorescent protein described in Nagal et al, *Nat. Biotechnol.,* 20:87-90 (2002). DsRed variants are described in, e.g., Shaner et al, *Nat. Biotechnol.,* 22:1567-1572 (2004), and include mStrawberry, mCherry, morange, mBanana, mHoneydew, and mTangerine. Additional DsRed variants are described in, e.g., Wang et al, *Proc. Natl. Acad. Sci. U.S.A.,* 101:16745-16749 (2004) and include mRaspberry and mPlum. Further examples of DsRed variants include mRFPmars described in Fischer et al, *FEBS Lett.,* 577:227-232 (2004) and mRFPruby described in Fischer et al, *FEBS Lett,* 580:2495-2502 (2006).

[00201] Amine-reactive and thiol-reactive fluorophores are available and generally used for labeling nucleotides and biomolecules. In some embodiments, nucleotides are fluorescently labeled during chemical synthesis, for example, incorporation of amines or thiols during

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nucleotide synthesis permit addition of fluorophores. Fluorescently labeled nucleotides are commercially available. For example, uridine and deoxyuridine triphosphates are available that are conjugated to ten different fluorophores that cover the spectrum.

[00202] In some embodiments, radioisotopes can be utilized as detectable molecules or detectable molecules for labeling nucleotides including, for example, ^{32}P, ^{33}P, ^{35}S, ^{3}H, and ^{125}I. These radioisotopes have different half-lives, types of decay, and levels of energy which can be tailored to match the needs of a particular experiment. For example, ^{3}H is a low energy emitter which results in low background levels, however this low energy also results in long time periods for autoradiography. Radioactively labeled ribonucleotides and deoxyribonucleotides are commercially available. Nucleotides are available that are radioactively labeled at the first, or α, phosphate group, or the third, or γ, phosphate group. For example, both [α-^{32}P]dATP and [γ-^{32}P]dATP are commercially available. In addition, different specific activities for radioactively labeled nucleotides are also available commercially and can be tailored for different experiments.

[00203] Suitable non-metallic isotopes include, but are not limited to, ^{11}C, ^{14}C, ^{13}N, ^{18}F, ^{123}I, ^{124}I, and ^{125}I. Suitable radioisotopes include, but are not limited to, ^{99}mTc, ^{95}Tc, ^{111}In, ^{62}Cu, ^{64}Cu, Ga, ^{68}Ga, and ^{153}Gd. Suitable paramagnetic metal ions include, but are not limited to, Gd(III), Dy(III), Fe(III), and Mn(II). Suitable X-ray absorbers include, but are not limited to, Re, Sm, Ho, Lu, Pm, Y, Bi, Pd, Gd, La, Au, Au, Yb, Dy, Cu, Rh, Ag, and Ir. In some embodiments, the radionuclide is bound to a chelating agent or chelating agent-linker attached to the aggregate. Suitable radionuclides for direct conjugation include, without limitation, ^{18}F, ^{124}I, ^{125}I, ^{131}I, and mixtures thereof. Suitable radionuclides for use with a chelating agent include, without limitation, ^{47}Sc, ^{64}Cu, ^{67}Cu, ^{89}Sr, ^{86}Y, ^{87}Y, ^{90}Y, ^{105}Rh, ^{111}Ag, ^{111}In, ^{117}mSn, ^{149}Pm, ^{153}Sm, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re, ^{211}At, ^{212}Bi, and mixtures thereof. Suitable chelating agents include, but are not limited to, DOTA, BAD, TETA, DTPA, EDTA, NTA, HDTA, their phosphonate analogs, and mixtures thereof. One of skill in the art will be familiar with methods for attaching radionuclides, chelating agents, and chelating agent-linkers to the particles.

[00204] Non-radioactive and non-fluorescent label monomers are also available. For example, biotin can be attached directly to nucleotides and detected by specific and high affinity binding to avidin or streptavidin which has been chemically coupled to an enzyme catalyzing a colorimetric reaction (such as phosphatase, luciferase, or peroxidase). Digoxigenin labeled

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nucleotides can also similarly be used for non-isotopic detection of nucleic acids. Biotinylated and digoxigenin-labeled nucleotides are commercially available.

[00205] In some embodiments, enzymatic reaction on a substrate can be utilized a detection method. In such embodiments, enzymes (e.g., horseradish peroxidase or alkaline phosphatase) can be linked to a nucleotide of a nucleic acid label and/or decoder probe. During detection, a substrate on which the particular enzyme reacts can be added to induce a colorimetric reaction. Any enzyme-substrate reactions known in the art can be used herein.

[00206] Very small particles, termed nanoparticles, also can be used as detectable labels or detectable molecules to label decoder probes or any nucleic acids. These particles range from 1-1000 nm in size and include diverse chemical structures such as gold and silver particles and quantum dots.

[00207] When irradiated with angled incident white light, silver or gold nanoparticles ranging from 40-120 nm will scatter monochromatic light with high intensity. The wavelength of the scattered light is dependent on the size of the particle. Four to five different particles in close proximity will each scatter monochromatic light which when superimposed will give a specific, unique color. Alternatively, the gold nanoparticles can be detected or "developed" using a silver-based developer such that the tiny nanoparticle can be detected easily, even with naked eyes. The particles are being manufactured by companies such as Genicon Sciences. Derivatized silver or gold particles can be generally attached to a broad array of molecular molecules including, proteins, antibodies, small molecules, receptor ligands, and nucleic acids. For example, the surface of the particle can be chemically derivatized to allow attachment to a nucleotide, which can then be incorporated into a decoder probe.

[00208] Another type of nanoparticle that can be used as a detectable molecule or detectable label are quantum dots. Quantum dots are fluorescing crystals 1-5 nm in diameter that are excitable by a large range of wavelengths of light. These crystals emit light, such as monochromatic light, with a wavelength dependent on their chemical composition and size. Quantum dots such as CdSe, ZnSe, InP, or InAs possess unique optical properties.

[00209] Due to their very small size the quantum dots can be generally coupled into oligonucleotides directly without affecting the solubility or use of the oligonucleotide. Thus, quantum dots can be coupled to decoder probes as described herein. To synthesize a decoder probe-quantum dot complex by conventional batch chemistry, both the decoder probe and the

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quantum dot require at least a reactive group of different kinds that can be reacted with each other. For example, if a decoder probe has an amino group and a quantum dot has an aldehyde group, these groups can react to form a Schiff base. A decoder probe can be derivatized to attach a single amino or other functional group using chemistry well known in the art. When a quantum dot is derivatized, the quantum dot can be covered with a chemical reagent which results in coating the entire surface of the nanoparticle with several functional groups.

[00210] A detectable molecule or detectable label can be attached to a nucleotide of a decoder probe or nucleic acid using a variety of methods well known in the art and described herein. For example, the detectable molecule or detectable label can be directly attached to the nucleotide in a 1:1 correspondence by incorporation of a radioactive phosphate into the phosphate backbone of the nucleotide. Also, for example, a general method for labeling phosphates with a fluorescent label that employs an imidazole derivative prepared from a BODIPY FL hydrazide has been reported (Wang and Giese, Anal. Chem. 65: 3518 (1993).

[00211] Depending on the labeling moiety used, it can be desirable to derivatize or chemically modify a nucleotide in order to bind the label monomer. These methods and chemistries are known in the art. In addition, a linker can be used to attach a detectable molecule or detectable label to a nucleotide in a 1:1 correspondence. For example, a fluorescently labeled nucleotide such as fluorescein-12-dUTP can have a fluorophore monomer attached via a four-atom aminoalkynyl group to the dUTP molecule.

[00212] These nucleotides attached to detectable molecules or detectable labels can be incorporated into a decoder probe or nucleic acid using several methods for labeling nucleic acids well known in the art. For example, enzymes such as DNA or RNA polymerases, Taq polymerases, terminal deoxynucleotidyl transferases, or reverse transcriptases can be used to incorporate labeled nucleotides into decoder probes or nucleic acids.

[00213] Labeled nucleotides can be incorporated into decoder probe sequences or nucleic acids, for example, by nick translation. In this procedure DNAse I is used to create single-strand nicks in double stranded DNA and then the 5′ to 3′ exonuclease and 5′ to 3′ polymerase actions of E. coli DNA polymerase I are used to remove stretches of single stranded DNA starting at the nicks and replace them with new strands made by incorporation of labeled nucleotides. Nick translation can utilize any labeled nucleotide including radioactively labeled nucleotides and biotinylated or digoxigenin labeled nucleotides. In a similar way T4 DNA polymerase can be

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used to incorporate labeled nucleotides. In addition, labeled nucleotides can be incorporated into nucleic acids using the polymerase chain reaction (PCR) and Taq polymerases. The degree of labeling can be controlled by including one, or up to all four labeled nucleotides. In addition, the degree of labeling can be controlled by increasing or decreasing the concentration of the labeled nucleotide(s).

[00214] Other methods for labeling decoder probes or nucleic acids include generating single-stranded cDNA from RNA by using a reverse transcriptase in the presence of labeled nucleotides. In addition, DNA can be cloned into a vector with SP6 or T7 polymerase sites. Transcription in the presence of SP6 or T7 RNA polymerase and labeled nucleotides results in a labeled RNA transcript. The transcript can be labeled to different degrees by including one or more labeled nucleotides. In addition, several nucleotides within a nucleic acid can be labeled, for example, by cloning DNA into a bacteriophage M13 based vector. Then the Klenow fragment of DNA polymerase I and the M13 universal probe primer can be used to synthesize the complementary stand with incorporation of labeled nucleotides.

[00215] Several methods are described above for incorporation of labeled nucleotides into newly synthesized decoder probes or nucleic acids. Existing nucleic acids can also be labeled using several methods known in the art. For example, RNA or DNA can be end-labeled with [γ-32P]ATP and T4 polynucleotide kinase. This kinase can be used to transfer the radioactive phosphate of ATP to a free 5′ OH group in either DNA or RNA. The enzyme also has a phosphatase activity and so two reactions are possible. In the forward reaction, the enzyme catalyzes phosphorylation following removal of 5′ terminal phosphates with alkaline phosphatase (or other phosphatase). In the exchange reaction, the kinase catalyzes the exchange of an existing 5′ phosphate with the third or γ phosphate of ATP. The latter reaction is carried out in the presence of excess ATP and ADP for efficient phosphorylation. Using this method the radioactive phosphate of ATP is transferred to the end of the nucleic acid molecule.

[00216] Decoder probes or nucleic acids can also be labeled with terminal deoxynucleotidyl transferase which adds labeled nucleotides onto the 3′ end of DNA fragments. Both single and double-stranded DNAs are substrates for this enzyme. The large (Klenow) fragment of E. coli DNA polymerase I can also be used to label the ends of decoder probes or nucleic acids. Since this enzyme has a 5′ to 3′ polymerase activity it can be used to "fill in" the 3′ ends of nucleic acid (e.g., DNA) fragments opposite of 5′ extensions or overhangs with labeled nucleotides. End-

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labeling of decoder probes or nucleic acids using polynucleotide kinase or terminal deoxynucleotidyl transferase results in the incorporation of one detectable label or detectable molecule per nucleic acid. The "fill in" reaction can be used to label the decoder probe or nucleic acid at one nucleotide per nucleic acid or at more than one nucleotide per nucleic acid.

[00217] In addition, decoder probes or nucleic acids can be labeled by modification of nucleotides within the nucleic acid sequences. For example, cytidine residues in DNA and RNA can be modified by reaction with sodium bisulfite to form sulfonate intermediates that can then be directly coupled to hydrazides or aliphatic amines. Virtually any of the fluorescent, biotin or other hydrazides or aliphatic amines can be used in this reaction. The bisulfite-activated cytidylic acid can also be coupled to aliphatic diamines such as ethylenediamine. The amine-modified nucleic acids (e.g., DNA or RNA) can then be modified with any of the amine-reactive dyes. In addition, phosphate groups can be targeted in nucleic acids for labeling. Although phosphate groups of nucleotides are not very reactive in aqueous solution, their terminal phosphate groups can react with carbodiimides and similar reagents in combination with nucleophiles to yield labeled phsophodiesters, phosphoramidates and phosphorothioates. For example, nucleic acids (e.g., DNA) can be reacted quantitatively with carbonyl diimidazole and a diamine such as ethylenediamine to yield a phosphoramidate that has a free primary amine and that this amine can then be modified with amino-reactive reagents. Fluorescent or biotinylated amines have been coupled to the 5′ phosphate of tRNA using dithiodipyridine and triphenylphosphine.

[00218] The bond between detectable molecules or detectable labels and decoder probes or nucleic acids can be covalent bonds or non-covalent bonds that are stable to hybridization. The detectable molecules or detectable labels can be bound to a decoder probe or a nucleic acid in a sequence specific manner, for example by the incorporation of a labeled nucleotide into a sequence that has been digested by a restriction enzyme. Alternatively the detectable molecules or detectable labels can be bound to a decoder probe or a nucleic acid in a non-sequence specific manner, for example by the incorporation of a label onto the terminal phosphate of a nucleic acid using [γ-^{32}P]ATP and T4 polynucleotide kinase.

Synthesis of nucleic acids (e.g., for at least part of the nucleic acid label such as pre-determined subsequences and/or decoder probe sequences

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[00219] The nucleic acids described herein can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the label attachment region and the annealed complementary polynucleotide sequences or segments, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the synthetic nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl)uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5′-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N-6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N2-carboxypropyl)uracil, (acp3)w, and 2,6-diaminopurine.

[00220] Alternatively, the synthetic nucleic acid can be produced biologically using a vector into which a nucleic acid has been subcloned. As one example, a linear single-stranded DNA backbone can be made from a double stranded plasmid DNA using a four step protocol that includes (i) linearization of the dsDNA with a restriction enzyme, (ii) dephosphorylation with a thermolabile phosphatase, (iii) digestion with a second restriction enzyme to separate the cloning vector from the backbone sequence, and (iv) digestion with a strand-specific lambda exonuclease digestion, leaving only one strand of the backbone fragment intact.

[00221] In various embodiments, the nucleic acids can be modified at the base moiety, sugar moiety or phosphate backbone to improve, e.g., the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acids can be modified to generate peptide nucleic acids (see Hyrup et al, 1996, Bioorganic & Medicinal Chemistry 40:5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, e.g., DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral

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backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup et al, 1996, Bioorganic & Medicinal Chemistry 4(1): 5-23; Perry-O'Keefe et al., 1996, Proc. Natl. Acad. Sci. USA 93: 14670-675.

[00222] In an exemplary embodiment, the selected novel nucleic acid sequence (e.g., DNA sequence) can be constructed synthetically as double-stranded nucleic acid by a commercial gene synthesis company and cloned in an oriented fashion into a "phagemid", a plasmid vector containing an M13 or f1 phage intergenic (IG) region which contains the cis-acting sequences necessary for nucleic acid (e.g., DNA) replication and phage encapsidation, such as pUC119. The appropriate orientation of the cloned insert relative to the phage origin of replication allows for the generation of a single-stranded DNA backbone which is the reverse complement of the RNA molecules generated by in vitro transcription for each label attachment region.

[00223] In order to generate the single-stranded nucleic acid (e.g., DNA) backbone (e.g., of at least part of the nucleic acid label and/or decoder probes), the phagemid is transformed into an E. coli strain containing an F′ episome. Subsequent infection of the transformed bacteria with a helper phage such as the M113 mutant K07 results in the secretion of the phagemid carrying the single-stranded nucleic acid sequence, packaged phage from which the circular, single-stranded nucleic acid is prepared using a standard protocol. This nucleic acid is linearized and the vector portion is excised by annealing short, complementary oligonucleotides to either end of the single-strander nucleic acid sequence to generate double-stranded restriction sites, followed by treatment with the appropriate restriction enzymes.

Analytes or target molecules

[00224] The terms "analyte," "target analyte," and "target molecule", as used interchangeably herein, refer to the molecule detected, identified or measured by binding of a detection reagent described herein whose probe reagent(s) recognize (i.e., are specific binding partners) thereto. In some embodiments, a target molecule or an analyte can be, but is not limited to, any of the following or any combinations of the following: nucleic acid, peptide, a polypeptide/protein (e.g., a bacterial or viral protein or an antibody), a lipid, a carbohydrate, a glycoprotein, a glycolipid, a small molecule, an organic monomer, sugar, peptidoglycan, a cell, a virus or a drug.

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Nucleic acids that can be analyzed by the methods herein include: double-stranded DNA, single-stranded DNA, single-stranded DNA hairpins, DNA/RNA hybrids, RNA (e.g. mRNA or miRNA) and RNA hairpins. Generally, a target molecule can be a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA. In other embodiments, a target molecule can be modified, e.g., by mutation or chemical reaction. In some embodiments, a target molecule can be synthetic or recombinant.

[00225] In some embodiments, an analyte can be an analyte comprising at least one post-translational modification, e.g., phosphorylations and/or glycosylations.

[00226] A target molecule or an analyte can be part of a sample that contains other components or can be the sole or major component of the sample. A target molecule or an analyte can be a component of a whole cell, tissue or body fluid, a cell or tissue extract, a fractionated lysate thereof or a substantially purified molecule. The target molecule can be present in solution or attached to a solid substrate, including, for example, to a solid surface such as a chip, microarray, bead or a blotting membrane. Also the target molecule or analyte can have either a known or unknown structure or sequence.

Sample

[00227] The methods, detection reagents and kits described herein can be used to analyze a sample from any sources, e.g., but not limited to biological samples (e.g., collected from organisms, animals or subjects), environmental samples, food, food byproduct, soil, archaeological samples, extraterrestrial samples, or any combinations thereof.

[00228] In some embodiments, the term "sample" refers to a biological sample. The term "biological sample" as used herein denotes a sample taken or isolated from a biological organism, e.g., tissue cell culture supernatant, cell lysate, a tissue sample (e.g., biopsy), a homogenate of a tissue sample from a subject or a fluid sample from a subject. Exemplary biological samples include, but are not limited to, blood, sputum, urine, cerebrospinal fluid, urine, sweat, mucus, nasal discharge, vaginal fluids, spinal fluid, pleural fluid, nipple aspirates, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, feces, sperm, cells or cell cultures, serum, leukocyte fractions, smears, tissue samples of all kinds, plants and parts of plants, microorganisms (such as bacteria), viruses (such as cytomegalo virus, HIV, hepatitis B, hepatitis C, hepatitis δ virus), yeasts, embryos, fungi, cell-

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free sample material, etc. The term also includes both a mixture of the above-mentioned samples such as fungus-infected plants or whole human blood containing mycobacteria as well as food samples that contain free or bound nucleic acids, or proteins, or cells containing nucleic acids or proteins, environmental samples which contain free or bound nucleic acids, or proteins, or cells containing nucleic acids or proteins. The term "biological sample" also includes untreated or pretreated (or pre-processed) biological samples.

[00229] A "biological sample" can contain cells from subject, but the term can also refer to non-cellular biological material, such as non-cellular fractions of blood, saliva, or urine, that can be used to measure gene expression levels. In some embodiments, the sample is from a resection, bronchoscopic biopsy, or core needle biopsy of a primary or metastatic tumor, or a cell block from pleural fluid. In addition, fine needle aspirate samples can be used. Samples can be either paraffin-embedded or frozen tissue. In some embodiments, a biological sample can comprise a biopsy, a surgically removed tissue, a swap, or any combinations thereof.

[00230] The sample can be obtained by removing a sample of cells from a subject, but can also be accomplished by using previously isolated cells (e.g. isolated by another person). In addition, the biological sample can be freshly collected or a previously collected sample. Furthermore, the biological sample can be utilized for the detection of the presence and/or quantitative level of a biomolecule of interest. Representative target analytes include, but are not limited to nucleic acids, proteins, and derivatives and fragments thereof.

[00231] In some embodiments, the biological sample is an untreated biological sample. As used herein, the phrase "untreated biological sample" refers to a biological sample that has not had any prior sample pre-treatment except for dilution and/or suspension in a solution. Exemplary methods for treating a biological sample include, but are not limited to, centrifugation, filtration, sonication, homogenization, heating, freezing and thawing, and any combinations thereof.

[00232] In accordance with some embodiments of various aspects described herein, a biological sample can be pre-processed, as described earlier, before employing the detection reagents and the methods described herein. In some embodiments, the biological sample can be filtered before isolating a cellular material according to the methods, apparatus and kits described herein.

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[00233] In some embodiments, the biological sample can be treated with a chemical and/or biological reagent. Chemical and/or biological reagents can be employed to protect and/or maintain the stability of the sample or target analytes during processing. In addition, or alternatively, chemical and/or biological reagents can be employed to release or expose target analytes from other components of the sample. One exemplary reagent is a protease inhibitor, which is generally used to protect or maintain the stability of the target analytes during processing.

[00234] In some embodiments, the term "sample" as used herein can refer to an environmental sample (including, but not limited to, air, agricultural (e.g., but not limited to hydrofarms or hydroponic samples), pond, water, wastewater, and soil samples); biological warfare agent samples; research samples including extracellular fluids. In some embodiments, an environmental sample can comprise a sample collected from a working surface of an equipment or machine (e.g., but not limited to, food or pharmaceutical product processing equipment or machine), a device (e.g., but not limited to, biomedical devices, implantation devices, fluid delivery devices such as a tubing, and/or a catheter), and/or a building or dwellings (e.g., but not limited to, food processing plants, pharmaceutical manufacturing plants, hospitals, and/or clinics).

[00235] In some embodiments, a sample can comprise food (e.g., solid and/or fluid food as well as processed food) and/or food byproduct. For example, the methods, detection reagents and kits described herein can be used to detect an analyte, e.g., a particular nutrient, in food and/or food byproduct, e.g., but not limited to, meat, milk, yoghurt, bread, starch-based products, vegetables, and any combinations thereof. In some embodiments, the methods, detection reagents and kits described herein can be used to detect a contaminant, e.g., bacteria, fungus, spores, molds, and/or viruses, in food and/or food byproduct.

[00236] In some embodiments, a sample can comprise a pharmaceutical product (e.g., but not limited to pills, tablets, gel capsules, syrups, vaccines, liquids, sprays, and any combinations thereof). For example, the methods, detection reagents and kits described herein can be used to detect the presence and/or measure the level of a particular active agent present in a pharmaceutical product. In some embodiments, the methods, detection reagents and kits described herein can be used to detect a contaminant, e.g., bacteria, fungus, spores, molds, and/or viruses, in a pharmaceutical product.

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[00237] In some embodiments, a sample can comprise an archaeological sample. In some embodiments, an archaeological sample can be obtained or collected from artifacts, architecture, biofacts (or ecofact, e.g., an object found at an archaeological site), cultural landscapes, and any combinations thereof.

[00238] In some embodiments, a sample can comprise an extraterrestrial sample. For example, an extraterrestrial sample can be any object or specimen (e.g., rock, meteorite, and/or environmental samples) obtained or collected from outer space or universe, and/or planets beyond the planet Earth, e.g., the moon, other planets (e.g., but not limited to, Mars, and/or Jupiter) and/or non-stellar objects.

Applications of the detection reagents

[00239] The detection reagents, compositions, methods and kits described herein can be used for diagnostic, prognostic therapeutic and screening purposes. The inventions described herein provide the advantage that many different target analytes can be analyzed at one time from a single sample using the methods described herein. This allows, for example, for several diagnostic tests to be performed on one sample.

[00240] When the probe reagent is a nucleic acid, the methods described herein can discriminate between nucleotide sequences. The difference between the target nucleotide sequences can be, for example, a single nucleic acid base difference, a nucleic acid deletion, a nucleic acid insertion, or rearrangement. Such sequence differences involving more than one base can also be detected. In some embodiments, the oligonucleotide probe sets have substantially the same length so that they hybridize to target nucleotide sequences at substantially similar hybridization conditions. As a result, the process described herein is able to detect various kinds of diseases or disorders, e.g., but not limited to, infectious diseases, genetic diseases, and cancer.

[00241] Without wishing to be bound, the detection reagents, compositions, methods and kits described herein can also be used for environmental monitoring, forensics, and food science. Examples of genetic analyses that can be performed on nucleic acids include e.g., SNP detection, STR detection, RNA expression analysis, promoter methylation, gene expression, virus detection, viral subtyping and drug resistance.

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[00242] In the area of environmental monitoring, some embodiments of various aspects described herein can be used for detection, identification, and monitoring of pathogenic and indigenous microorganisms in natural and engineered ecosystems and microcosms such as in municipal waste water purification systems and water reservoirs or in polluted areas undergoing bioremediation. It can also be used to detect plasmids containing genes that can metabolize xenobiotics, to monitor specific target microorganisms in population dynamic studies, or either to detect, identify, or monitor genetically modified microorganisms in the environment and in industrial plants.

[00243] Some embodiments of various aspects described herein can also be used in a variety of forensic areas, including for human identification for military personnel and criminal investigation, paternity testing and family relation analysis, HLA compatibility typing, and screening blood, sperm, or transplantation organs for contamination.

[00244] In the food and feed industry, some embodiments of various aspects described herein have a wide variety of applications. For example, it can be used for identification and characterization of production organisms such as yeast for production of beer, wine, cheese, yogurt, bread, etc. Another area of use is related to the quality control and certification of products and processes (e.g., livestock, pasteurization, and meat processing) for contaminants. Other uses include the characterization of plants, bulbs, and seeds for breeding purposes, identification of the presence of plant-specific pathogens, and detection and identification of veterinary infections.

[00245] In some embodiments, the methods, detection reagents and kits described herein can be used to detect the presence and/or measure the level of a particular active agent present in a pharmaceutical product. In some embodiments, the methods, detection reagents and kits described herein can be used to detect a contaminant, e.g., bacteria, fungus, spores, molds, and/or viruses, in a pharmaceutical product.

[00246] In some embodiments, the methods, detection reagents and kits described herein can be used to detect the presence and/or measure the level of an analyte of interest present in an archaeological sample, e.g., obtained or collected from artifacts, architecture, biofacts (or ecofact, e.g., an object found at an archaeological site), cultural landscapes, and any combinations thereof.

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[00247] In some embodiments, the methods, detection reagents and kits described herein can be used to detect the presence and/or measure the level of an analyte of interest present in an extraterrestrial sample, e.g., any object or specimen (e.g., rock, meteorite, and/or environmental samples) obtained or collected from outer space or universe, and/or planets beyond the planet Earth, e.g., the moon, other planets (e.g., but not limited to, Mars, and/or Jupiter) and/or non-stellar objects. For example, the methods, detection reagents, and kits described herein can be used to analyze the composition of an extratrerresterial sample or specimen, e.g., a rock specimen, a water sample, and/or an air sample.

[00248] In some embodiments, the detection reagents and methods described herein can be used to detect and/or identify analytes comprising a post-translation modification, e.g., phosphorylation or glycosylation. As such, in some embodiments, the detection reagents and methods described herein can differentiate between multiple different glycosylation forms of recombinant therapeutic proteins

[00249] ***Immunohistochemistry***: Some embodiments of various aspects described herein can be used to perform antibody-based staining of cell or tissues for microscopic evaluation. This can involve either unfixed or unpermeabilized samples examined for surface or extracellular antigens, or permeabilized samples wherein intracellular antigens are also probed. While very common, immunohistochemistry is typically limited to probing with a small set of antibodies at a time (due to the limitation of available optical colors), and multiple staining cycles are generally avoided, since the stripping of the preceding cycle's antibodies can damage the sample. Some embodiments of the detection reagents comprising antibodies as probe reagents and nucleic acid labels can allow for many antibodies to be used concurrently, which saves time and sample material, extract more data, and demand less prior knowledge of the sample. In some embodiments, without wishing to be bound by theory, when a subset of the probed antigens can overlap spatially, the detection reagents with a plurality of contiguous pre-determined subsequences (e.g., each pre-determined subsequences contain one nucleotide, as shown in **Fig. 1**) can be used.

[00250] ***In-situ hybridization:*** Fluorescence in-situ hybridization (FISH) is a technique for detecting (and/or quantifying) the presence of certain cellular DNA or RNA (often ribosomal RNA). As with other fluorescence-based techniques, FISH is limited to the number of colors available to the microscopy. Using some embodiments of the detection reagents and/or methods

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described herein, the cells can be probed for many sequences simultaneously, thus allowing the user to save time and sample material, extract more data, and demand less prior knowledge of the sample.

[00251] ***Expression profiling***: Nucleic-acid probe reagents of the detection reagents can target messenger RNA or micro RNA and provide information on the RNA-level expression state of the cell. Using some embodiments of the detection reagents and/or methods described herein, the assay can capture and probe numerous mRNA and/or miRNA at once, reducing and/or eliminating potentially harmful stripping steps.

[00252] ***Western blots:*** Western blots are protein assays wherein proteins are separated electrophoretically, transferred to a membrane and stained. In many cases, the staining is done using one or a few antibodies in order to detect the corresponding antigens in the blot. By using some embodiments of the detection reagents and/or methods described herein, the blot can be simultaneously probed using a large number of antibodies without antibody-stripping steps. This can allow one blot to provide significantly more information than the conventional Western blots where one antibody is usually probed at a time and can require antibody stripping for the next antibody probing, thereby saving sample material and time.

[00253] ***Diagnostic/Prognostic Methods:*** The present methods can be applied to the analysis of biological samples obtained or derived from a patient so as to determine whether a diseased cell type is present in the sample and/or to stage the disease.

[00254] In some embodiments, the methods described herein are used in the diagnosis of a condition. As used herein the term "diagnose" or "diagnosis" of a condition includes predicting or diagnosing the condition, determining predisposition to the condition, monitoring treatment of the condition, diagnosing a therapeutic response of the disease, and prognosis of the condition, condition progression, and response to particular treatment of the condition. For example, a blood sample can be assayed according to any of the methods described herein to determine the presence and/or quantity of markers of a cancerous cell type in the sample, thereby diagnosing or staging the cancer.

[00255] In some embodiments, the detection reagents and methods described herein can be directly used as contrast agents for in vivo or in situ diagnosis, in which detection reagents are administered to a subject, either by oral administration or local injection. The probe reagents of the detection reagents can bind to the target analytes, e.g., biomarkers for specific diseases or

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disorders, and detection of the nucleic acid labels using the methods described herein can then locate the target analytes. In some embodiments, the detection reagents and methods described herein can be used to locate microscopic tumors in a subject, where other conventional methods are not sensitive enough to detect such microscopic tumors.

[00256] Cancers which can be detected by some embodiments of the process described herein generally involve oncogenes, tumor suppressor genes, or genes involved in DNA amplification, replication, recombination, or repair. Examples of these include: BRCA1 gene, p53 gene, APC gene, Her2/Neu amplification, Bcr/Ab1, K-ras gene, and human papillomavirus Types 16 and 18. Some embodiments of various aspects can be used to identify amplifications, large deletions as well as point mutations and small deletions/insertions of the above genes in the following common human cancers: leukemia, colon cancer, breast cancer, lung cancer, prostate cancer, brain tumors, central nervous system tumors, bladder tumors, melanomas, liver cancer, osteosarcoma and other bone cancers, testicular and ovarian carcinomas, head and neck tumors, and cervical neoplasms.

[00257] Genetic diseases can also be detected by some embodiments of the process described herein. This can be carried out by prenatal or post-natal screening for chromosomal and genetic aberrations or for genetic diseases. Examples of detectable genetic diseases include: 21 hydroxylase deficiency, cystic fibrosis, Fragile X Syndrome, Turner Syndrome, Duchenne Muscular Dystrophy, Down Syndrome or other trisomies, heart disease, single gene diseases, HLA typing, phenylketonuria, sickle cell anemia, Tay-Sachs Disease, thalassemia, Klinefelter Syndrome, Huntington Disease, autoimmune diseases, lipidosis, obesity defects, hemophilia, inborn errors of metabolism, and diabetes.

[00258] Alternatively, the methods described herein can be used to diagnose pathogen infections, for example infections by intracellular bacteria and viruses, by determining the presence and/or quantity of markers of bacterium or virus, respectively, in the sample.

[00259] A wide variety of infectious diseases can be detected by some embodiments of the process described herein. Typically, these are caused by bacterial, viral, parasite, and fungal infectious agents. The resistance of various infectious agents to drugs can also be determined using some embodiments of various aspects described herein.

[00260] Bacterial infectious agents which can be detected by some embodiments of various aspects described herein can include Escherichia coli, Salmonella, Shigella, Klebsiella,

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Pseudomonas, Listeria monocytogenes, Mycobacterium tuberculosis, Mycobacterium aviumintracellulare, Yersinia, Francisella, Pasteurella, Brucella, Clostridia, Bordetella pertussis, Bacteroides, Staphylococcus aureus, Streptococcus pneumonia, B-Hemolytic strep., Corynebacteria, Legionella, Mycoplasma, Ureaplasma, Chlamydia, Neisseria gonorrhea, Neisseria meningitides, Hemophilus influenza, Enterococcus faecalis, Proteus vulgaris, Proteus mirabilis, Helicobacter pylori, Treponema palladium, Borrelia burgdorferi, Borrelia recurrentis, Rickettsial pathogens, Nocardia, and Acitnomycetes.

[00261] Fungal infectious agents which can be detected by some embodiments of various aspects described herein can include Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Candida albicans, Aspergillus fumigautus, Phycomycetes (Rhizopus), Sporothrix schenckii, Chromomycosis, and Maduromycosis.

[00262] Viral infectious agents which can be detected by some embodiments of various aspects described herein can include human immunodeficiency virus, human T-cell lymphocytotrophic virus, hepatitis viruses (e.g., Hepatitis B Virus and Hepatitis C Virus), Epstein-Barr Virus, cytomegalovirus, human papillomaviruses, orthomyxo viruses, paramyxo viruses, adenoviruses, corona viruses, rhabdo viruses, polio viruses, toga viruses, bunya viruses, arena viruses, rubella viruses, and reo viruses.

[00263] Parasitic agents which can be detected by some embodiments of various aspects described herein can include Plasmodium falciparum, Plasmodium malaria, Plasmodium vivax, Plasmodium ovale, Onchoverva volvulus, Leishmania, Trypanosoma spp., Schistosoma spp., Entamoeba histolytica, Cryptosporidum, Giardia spp., Trichimonas spp., Balatidium coli, Wuchereria bancrofti, Toxoplasma spp., Enterobius vermicularis, Ascaris lumbricoides, Trichuris trichiura, Dracunculus medinesis, trematodes, Diphyllobothrium latum, Taenia spp., Pneumocystis carinii, and Necator americanis.

[00264] In some embodiments, contacting a biological sample with the detection reagents described herein immune-staining, in-situ hybridization or a combination, can be used for the purpose of detecting and identifying pathogens in biological samples. In such embodiments, for example, surface or intracellular antigens, DNA, ribosomal RNA and/or messenger RNA can be detected with some embodiments of the detection reagents and methods described herein. Detection reagents and methods described herein can be especially useful in this context since a)

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which pathogen may be in the sample is not known in advance, and b) each pathogen cell should respond to one or few of the probes, facilitating the detection reagent and nucleic acid label design.

[00265] Some embodiments of various aspects described herein can also be used for detection of drug resistance by infectious agents. For example, vancomycin-resistant Enterococcus faecium, methicillin-resistant Staphylococcus aureus, penicillin-resistant Streptococcus pneumoniae, multi-drug resistant Mycobacterium tuberculosis, and AZT-resistant human immunodeficiency virus can all be identified with some embodiments of various aspects described herein.

[00266] Thus, the target molecules detected using the compositions and methods described herein can be either patient markers (such as a cancer marker) or markers of infection with a foreign agent, such as bacterial or viral markers.

[00267] Because of the quantitative nature of detection reagents, the compositions and methods described herein can be used to quantitate target molecules whose abundance is indicative of a biological state or disease condition, for example, blood markers that are upregulated or downregulated as a result of a disease state.

[00268] In addition, the compositions and methods described herein can be used to provide prognostic information that assists in determining a course of treatment for a patient. For example, the amount of a particular marker for a tumor can be accurately quantified from even a small sample from a patient. For certain diseases like breast cancer, overexpression of certain genes, such as Her2-neu, indicate a more aggressive course of treatment will be needed.

[00269] In some embodiments, the compositions and methods described herein can be administered to a subject for in vivo diagnosis and/or monitoring of a disease or disorder. Specific devices or methods known in the art for the in vivo detection of fluorescence, e.g., from fluorophores or fluorescent proteins, include, but are not limited to, in vivo near-infrared fluorescence (see, e.g., Frangioni, *Curr. Opin. Chem. Biol,* 7:626-634 (2003)), the MaestroTM in vivo fluorescence imaging system (Cambridge Research & Instrumentation, Inc.; Woburn, Mass.), in vivo fluorescence imaging using a flying-spot scanner (see, e.g., Ramanujam et al, *IEEE Transactions on Biomedical Engineering,* 48:1034-1041 (2001), and the like. Other methods or devices for detecting an optical response include, without limitation, visual inspection, CCD cameras, video cameras, photographic film, laser-scanning devices,

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fluorometers, photodiodes, quantum counters, epifluorescence microscopes, scanning microscopes, flow cytometers, fluorescence microplate readers, or signal amplification using photomultiplier tubes.

[00270] Any device or method known in the art for detecting the radioactive emissions of radionuclides in a subject is suitable for use in various aspects described herein. For example, methods such as Single Photon Emission Computerized Tomography (SPECT), which detects the radiation from a single photon gamma-emitting radionuclide using a rotating gamma camera, and radionuclide scintigraphy, which obtains an image or series of sequential images of the distribution of a radionuclide in tissues, organs, or body systems using a scintillation gamma camera, may be used for detecting the radiation emitted from a radiolabeled aggregate. Positron emission tomography (PET) is another suitable technique for detecting radiation in a subject.

[00271] ***Analysis of Pathology Samples:*** RNA extracted from formaldehyde- or paraformaldehyde-fixed paraffin-embedded tissue samples is typically poor in quality (fragmented) and low in yield. This makes gene expression analysis of low-expressing genes in histology samples or archival pathology tissues extremely difficult and often completely infeasible. The detection reagents and methods described herein can fill this unmet need by allowing the analysis of very small quantities of low-quality total RNA.

[00272] To use detection reagents in such an application, total RNA can be extracted from formaldehyde- or paraformaldehyde-fixed paraffin-embedded tissue samples (or similar) using commercially available kits such as RecoverAll Total Nucleic Acid Isolation Kit (Ambion) following manufacturer's protocols. RNA in such samples is frequently degraded to small fragments (200 to 500 nucleotides in length), and many paraffin-embedded histology samples only yield tens of nanograms of total RNA. Small amounts (5 to 100 ng) of this fragmented total RNA can be used directly as target analyte upon contact with the detection reagents described herein, using the methods described herein described herein.

[00273] ***Screening Methods:*** The methods described herein can be used, among other things, for determining the effect of a perturbation, including chemical compounds, mutations, temperature changes, growth hormones, growth factors, disease, or a change in culture conditions, on various target molecules, thereby identifying target molecules whose presence, absence or levels are indicative of particular biological states. In one embodiment, some aspects described herein can be used to elucidate and discover components and pathways of disease

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states. For example, the comparison of quantities of target molecules present in a disease tissue with "normal" tissue allows the elucidation of important target molecules involved in the disease, thereby identifying targets for the discovery/screening of new drug candidates that can be used to treat disease.

[00274] ***Targeted Delivery Vehicles***: In some embodiments, the therapeutic and/or diagnostic agent can be encapsulated within the particles that provide conjugation sites for the probe reagents and nucleic acid labels. In such embodiments, the detection reagents and methods described herein can be used to deliver a therapeutic and/or diagnostic agent to cells where the probe reagents of the detection reagents bind. Nucleic acid labels of the detection reagents can be detected to monitor the location of drug administration.

[00275] ***Cell Lineage Tracking***: In some embodiments, the detection reagents described herein can be used to track cell lineage, e.g., in culture or in vivo. For example, the probe reagents of the detection reagents can bind to live cells of interest, allowing labeling and/or tracking of individual cell differentiation. Examples of such application include, but are not limited to, lineage tracking in stem cell differentiation, and lineage determination of dendritic cells and/or white blood cells. In some embodiments, these cells after lineage determination can be further used accordingly.

Kits comprising detection reagents

[00276] Kits for various assays are also provided herein. In some embodiments, a kit can comprise: (a) a plurality of nucleic acid labels of the detection reagents described herein; and (b) at least one coupling reagent required to conjugate the nucleic acid labels to probe reagents of interest. In such embodiments, users can attach the provided nucleic acid labels to their probe reagents of interest to form their own detection reagents described herein. Examples of the coupling reagent required for nucleic acid label-probe reagent conjugation can include any reagents that are generally used to perform any of the conjugation methods described herein, e.g., biotin and avidin-like molecules such as streptavidin or neutravidin. However, in alternative embodiments, the kit can comprise a plurality of the detection reagents described herein that are ready for use and thus no nucleic acid label-probe reagent conjugation steps are required to be performed by users prior to use.

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[00277] In some embodiments, the kit can further comprise at least one reagent, e.g., used in a readout method described herein. For example, if the readout method is sequencing-based, the kit can further comprise at least one agent used in sequencing-based readout. Alternatively, if the readout method is hybridization-based, the kit can further comprise at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.

[00278] Accordingly, in some embodiments, a kit for hybridization-based readout can comprise: (a) a plurality of nucleic acid labels of the detection reagents described herein; (b) at least one reagent required to conjugate the nucleic acid labels to probe reagents of interest; and (c) at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature. In some embodiments, the kit can further comprise at least one reagent, e.g., used in the readout method.

[00279] In alternative embodiments, a kit for hybridization-based readout can comprise: (a) a plurality of the detection reagents described herein; (b) at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature; and (c) at least one reagent.

[00280] In any embodiments of the kit described herein, the plurality of the detection reagents can include one or more different kinds of the detection reagents. In some embodiments, it is desirable to provide different kinds of the detection reagents (e.g., different types of probe reagents, target binding domains, and/or target analytes) in separate containers. The number of different kinds of the detection reagents provided in the kit can be tailored for each application described herein. In some embodiments, the kit can comprise at least 2, at least 3, at least 4, at least 5, at least 6 at least 7, at least 8, at least 9, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100 or more different kinds of the detection reagents.

[00281] In any embodiments of the kit described herein , the kit can comprise a plurality of sets of decoder probes, e.g., e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10 or more sets of decoder probes, wherein each set of decoder probes can target a different pre-determined sequence of the nucleic acid

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label. In each set of the decoder probes, there can be about 2, 3, 4, 5, 6, 7, 8, 9, 10 or more distinct populations of decoder probes. The decoder probes can be pre-labeled with at least one detectable label or unlabeled. In some embodiments where the decoder probes are not pre-labeled, the kit can further comprise one or more distinct detectable labels provided in separate containers for labeling the decoder probes. In some embodiments, the detectable label can be an optical label described herein.

[00282] Examples of a reagent, e.g., used in a readout method, can include, but are not limited to, a readout reagent, a wash buffer, a signal removal buffer, buffers for performing hybridization reactions, restriction endonucleases, nucleic acid ligases, and any combinations thereof.

[00283] In some embodiments, the detection reagents can be provided in a solution phase. In other embodiments, the detection reagents can be immobilized in a solid support or substrate.

[00284] By "substrate" or "solid support" or other grammatical equivalents herein is meant any material that can be modified to contain discrete individual sites appropriate for the attachment or association of beads and is amenable to at least one detection method. As will be appreciated by those in the art, the number of possible substrates is very large. Possible substrates include, but are not limited to, glass and modified or functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, Teflon, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, optical fiber bundles, and a variety of other polymers. In general, the substrates allow optical detection and do not themselves appreciably fluoresce.

[00285] Generally the substrate is flat (planar), although as will be appreciated by those in the art, other configurations of substrates may be used as well; for example, three dimensional configurations can be used, for example by embedding the detection reagents in a porous block of plastic that allows sample access to the detection reagents and using a confocal microscope for detection. In some embodiments, the detection reagents can be placed on the inside surface of a tube, for flow-through sample analysis to minimize sample volume. Preferred substrates include optical fiber bundles, and flat planar substrates such as glass, polystyrene and other plastics and acrylics. In some embodiment, the solid support or substrate can be a multi-well plate.

[00286] In addition to the above mentioned components, the kit can include informational material. The informational material can be descriptive, instructional, marketing or other

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material that relates to the methods described herein and/or the use of the aggregates for the methods described herein. For example, the informational material describes methods for administering the detection reagents to a subject, and/or includes instructions to label decoder probes with detectable labels provided therein and/or instructions to conjugate at least one nucleic acid label to a probe reagent. The kit can also include a delivery device.

[00287] In some embodiments, the informational material can include instructions to administer the formulation in a suitable manner, e.g., in a suitable dose, dosage form, or mode of administration (e.g., a dose, dosage form, or mode of administration described herein). In another embodiment, the informational material can include instructions for identifying a suitable subject, e.g., a human, e.g., an adult human. The informational material of the kits is not limited in its form. In many cases, the informational material, e.g., instructions, is provided in printed matter, e.g., a printed text, drawing, and/or photograph, e.g., a label or printed sheet. However, the informational material can also be provided in other formats, such as Braille, computer readable material, video recording, or audio recording. In another embodiment, the informational material of the kit is a link or contact information, e.g., a physical address, email address, hyperlink, website, or telephone number, where a user of the kit can obtain substantive information about the formulation and/or its use in the methods described herein. Of course, the informational material can also be provided in any combination of formats.

[00288] In some embodiments, the kit contains separate containers, dividers or compartments for each component and informational material. For example, each different component can be contained in a bottle, vial, or syringe, and the informational material can be contained in a plastic sleeve or packet. In other embodiments, the separate elements of the kit are contained within a single, undivided container. For example, the formulation is contained in a bottle, vial or syringe that has attached thereto the informational material in the form of a label.

[00289] In some embodiments, the kit includes a plurality, e.g., a pack, of individual containers, each containing one or more unit dosage forms of the composition comprising the detection reagents. For example, the kit includes a plurality of syringes, ampules, foil packets, or blister packs, each containing a single unit dose of the formulation. The containers of the kits can be air tight and/or waterproof.

[00290] *Embodiments of the various aspects described herein can be illustrated by the following numbered paragraphs.*

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1. A method for detecting a plurality of analytes in a sample, comprising:

    a. contacting the sample with a composition comprising a plurality of detection reagents, wherein each subpopulation of the detection reagents targets at least one different analyte, and wherein each detection reagent comprises:

        at least one probe reagent targeting an analyte and at least one nucleic acid label comprising one or a plurality of pre-determined subsequences, wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together; and wherein at least a portion of said one or the plurality of pre-determined subsequences form an identifier of said at least one probe reagent; and

    b. detecting in a temporally-sequential manner said one or the plurality of the pre-determined subsequences of said detection reagent, wherein said detection of the subsequences each generates a signal signature corresponding to said subsequence, and wherein a temporal order of the signal signatures corresponding to said one or the plurality of the subsequences of said detection reagent identifies a subpopulation of the detection reagents.

2. The method of paragraph 1, wherein said each subpopulation of the detection reagents targets a set of analytes (e.g., at least two analytes or more).

3. The method of paragraph 1 or 2, wherein the temporal order of the signal signatures corresponding to said one or the plurality of the subsequences of said detection reagent is unique for each subpopulation of the detection reagents.

4. The method of any of paragraphs 1-3, wherein said detection reagents are present in a soluble phase.

5. The method of any of paragraphs 1-4, further comprising processing said sample before said contacting with said plurality of detection reagents.

6. The method of any of paragraphs 1-5, further comprising removing any unbound detection reagents before the detecting step (b).

7. The method of any of paragraphs 1-6, further comprising comparing said temporal order of the signal signatures with different identifiers of said at least one probe reagent,

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wherein an agreement between the temporal order of the signal signatures and a particular identifier of said at least one probe reagent identifies the analyte in the sample.

8. The method of any of paragraphs 1-7, further comprising measuring the intensity of the signal signatures generated from each subpopulation of the detection reagents.

9. The method of paragraph 8, wherein the intensity of the signal signatures generated from each subpopulation of the detection reagents indicates an amount of the analyte.

10. The method of paragraph 8 or 9, wherein the intensity of the signal signatures generated from each subpopulation of the detection reagents is used in identification of the subpopulation of the detection reagents.

11. The method of any of paragraphs 1-10, wherein said detecting of step (b) comprises sequencing.

12. The method of paragraph 11, wherein said sequencing is performed via ligation, hybridization, synthesis, amplification, or single-base extension.

13. The method of any of paragraphs 1-12 wherein said detecting of step (b) comprises hybridizing a decoder probe with said subsequence, wherein said decoder probe comprises a detectable label.

14. The method of any of paragraphs 1-13, wherein said detecting of step (b) comprises:

    a. hybridizing a set of decoder probes with a subsequence of the detection reagents, wherein each subpopulation of the decoder probes comprises a detectable label, each detectable label producing a signal signature;

    b. detecting said signal signature produced by the hybridization of said set of decoder probes;

    c. optionally removing said different signal signature produced by the hybridization of said set of decoder probes; and

    d. repeating steps (a) through (c) for other subsequences of said detection reagents, thereby producing a temporal order of the signal signatures corresponding to said each detection reagent.

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15. The method of paragraph 14, wherein said each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.

16. The method of paragraph 14 or 15, wherein said each subpopulation of the decoder probes is at least partially or completely complementary to said subsequence of the detection reagents.

17. The method of any of paragraphs 14-16, wherein at least two or more subpopulations of the decoder probes are at least partially or completely complementary to the same subsequence of the detection reagents.

18. The method of any of paragraphs 14-17, wherein said removing step is performed by washing, heating, photobleaching, displacement, cleavage, enzymatic digestion, quenching, chemical degradation, bleaching, oxidation or any combinations thereof.

19. The method of any of paragraphs 14-18, wherein said detectable label comprises or is an optical label selected from the group consisting of a small-molecule dye, a fluorescent molecule, a fluorescent protein, a quantum dot, Raman label, a chromophore, and any combinations thereof.

20. The method of any of paragraphs 14-19, wherein said detectable label comprises or is a colorimetric reagent.

21. The method of any of paragraphs 14-20, wherein said detectable label comprises or is a Raman label.

22. The method of any of paragraphs 1-21, wherein said signal signatures comprise or are optical signatures.

23. The method of paragraph 22, wherein said optical signatures comprise signatures of fluorescent color, visible light, no-color, Raman label, or any combinations thereof.

24. The method of paragraph 22, wherein said optical signatures comprise signatures of one or more fluorescent colors, one or more visible lights, one or more no-colors, one or more Raman labels, or any combinations thereof.

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25. The method of any of paragraphs 22-24, wherein said optical signatures are detected by optical imaging or spectroscopy.

26. The method of any of paragraphs 1-25, wherein said analytes are selected from the group consisting of antigens, receptors, proteins, peptides, sugars, glycoproteins, peptidoglycans, lipids, nucleic acids, oligonucleotides, cells, viruses, and any combinations thereof.

27. The method of any of paragraphs 1-26, wherein said nucleic acids are selected from the group consisting of cellular DNA or RNA, messenger RNA, microRNA, ribosomal RNA, and any combinations thereof.

28. The method of any of paragraphs 1-27, wherein said sample is a protein sample immobilized on a solid support.

29. The method of paragraph 28, wherein the solid support is a blotting membrane.

30. The method of any of paragraphs 1-27, wherein said sample is a biological sample.

31. The method of paragraph 30, wherein said biological sample comprises one or more cells, one or more tissues, one or more fluids or any combinations thereof.

32. The method of paragraph 30 or 31, wherein said biological sample comprises blood, sputum, cerebrospinal fluid, urine, saliva, sperm, sweat, mucus, nasal discharge, vaginal fluids or any combinations thereof.

33. The method of any of paragraphs 30-31, wherein the said biological sample comprises a biopsy, a surgically removed tissue, a swap or any combinations thereof.

34. The method of paragraphs 1-27, wherein said sample comprises an environmental sample, food, food byproduct, soil, an archaeological sample, an extraterrestrial sample, or any combinations thereof.

35. The method of any of paragraphs 1-34, wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together by at least one linker.

36. The method of paragraph 35, wherein said linker is a bond.

37. The method of paragraph 35 or 36, wherein said linker is a linker molecule.

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38. The method of paragraph 37, wherein said linker molecule is a polymer, sugar, nucleic acid, peptide, protein, hydrocarbon, lipid, polyethylene glycol, crosslinker, or any combinations thereof.

39. The method of any of paragraphs 35-38, wherein said linker is a particle.

40. The method of paragraph 39, wherein said particle is selected from a group consisting of a gold nanoparticle, a magnetic bead or nanoparticle, a polystyrene bead, a nanotube, a nanowire, a microparticle, and any combinations thereof.

41. The method of paragraph 40, wherein said particle is a nanoparticle.

42. The method of any of paragraphs 39-41, wherein said particle is modified.

43. The method of any of paragraphs 39-42, wherein said particle is coated with streptavidin or a derivative thereof.

44. The method of any of paragraphs 39-43, wherein said particle is modified with at least one functional group.

45. The method of paragraph 44, wherein said at least one functional group is selected from the group consisting of amine, carboxyl, hydroxyl, aldehyde, ketone, tosyl, silanol, chlorine, hydrazine, hydrazide, photoreactive groups, and any combinations thereof.

46. The method of any of paragraphs 35-45, wherein said linker is multivalent.

47. The method of paragraph 46, wherein when the multivalent linker is an avidin-like molecule, both the probe reagent and the nucleic acid label are biotinylated.

48. The method of any of paragraphs 1-47, wherein said at least one probe reagent is selected from the group consisting of a nucleic acid, an antibody or a portion thereof, an antibody-like molecule, an enzyme, a cell, an antigen, a small molecule, a protein, a peptide, a peptidomimetic, a sugar, a carbohydrate, a lipid, a glycan, a glycoprotein, an aptamer, and any combinations thereof.

49. The method of any of paragraphs 1-48, wherein said at least one probe reagent is modified.

50. The method of any of paragraphs 1-49, wherein said at least one probe reagent is biotinylated.

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51. The method of any of paragraphs 1-50, wherein said at least one nucleic acid label is single-stranded, double-stranded, partially double-stranded, a hairpin, linear, circular, branched, a concatemer, or any combinations thereof.

52. The method of any of paragraphs 1-51, wherein said at least one nucleic acid label is modified.

53. The method of any of paragraphs 1-52, wherein said at least one nucleic acid label is designed for minimal cross-hybridization of bases with each other.

54. The method of any of paragraphs 1-53, wherein said at least one nucleic acid label is conjugated to at least one detectable molecule.

55. The method of paragraph 54, wherein said at least one detectable molecule is an optical molecule selected from the group consisting of a small-molecule dye, a fluorescent protein, a quantum dot, a Raman label, a chromophore, and any combinations thereof.

56. The method of any of paragraphs 1-55, wherein each of said plurality of the pre-determined subsequences comprises at least one base.

57. The method of any of paragraphs 1-56, wherein each of said plurality of the pre-determined subsequences comprises from 1 to 100 nucleobases.

58. The method of any of paragraphs 1-57, wherein said plurality of the pre-determined subsequences are conjugated together by at least one sequence linker.

59. The method of paragraph 58, wherein said sequence linker is a bond.

60. The method of any of paragraphs 58-59, wherein said sequence linker is a nucleotidic linker.

61. The method of paragraph 60, wherein said nucleotidic linker is single-stranded, double-stranded, partially double-stranded, a hairpin or any combinations thereof.

62. The method of paragraph 60 or 61, wherein said nucleotidic linker is at least one nucleotide long.

63. The method of any of paragraphs 1-62, wherein said detection reagent comprises one probe reagent and a plurality of nucleic acid labels.

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64. The method of any of paragraphs 1-62, wherein said detection reagent comprises a plurality of probe reagents and a nucleic acid label.

65. The method of any of paragraphs 1-62, wherein said detection reagent comprises a plurality of probe reagents and a plurality of nucleic acid labels.

66. The method of any of paragraphs 1-65, wherein the method is adapted for use in immunofluorescence.

67. The method of any of paragraphs 1-66, wherein the method is adapted for use in immunohistochemistry.

68. The method of any of paragraphs 1-67, wherein the method is adapted for use in fluorescence in situ hybridization.

69. The method of any of paragraphs 1-68, wherein the method is adapted for use in western blot.

70. A detection reagent comprising at least one probe reagent and at least one nucleic acid label,

wherein said at least one nucleic acid label comprises at least one pre-determined subsequence to be detected in a temporally-sequential manner;

wherein said at least one pre-determined subsequence forms an identifier of said at least one probe reagent; and

wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together.

71. The detection reagent of paragraph 70, wherein the detection reagent is present in a soluble phase.

72. The detection reagent of paragraph 70 or 71, wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together by at least one linker.

73. The detection reagent of paragraph 72, wherein said linker is a bond.

74. The detection reagent of paragraph 72 or 73, wherein said linker is a linker molecule.

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75. The detection reagent of paragraph 74, wherein said linker molecule is a polymer, sugar, nucleic acid, peptide, protein, hydrocarbon, lipid, polyethelyne glycol, crosslinker or combination thereof.

76. The detection reagent of any of paragraphs 72-75, wherein said linker is a particle.

77. The detection reagent of paragraph 76, wherein said particle is selected from a group consisting of a gold nanoparticle, a magnetic bead or nanoparticle, a polystyrene bead, a nanotube, a nanowire, a microparticle, and any combinations thereof.

78. The detection reagent of paragraph 76 or 77, wherein said particle is a nanoparticle.

79. The detection reagent of any of paragraphs 76-78, wherein said particle is modified.

80. The detection reagent of any of paragraphs 76-79, wherein said particle is coated with streptavidin or a derivative thereof.

81. The detection reagent of any of paragraphs 76-80, wherein said particle is modified with at least one functional group.

82. The detection reagent of paragraph 81, wherein the at least one functional group is selected from the group consisting of amine, carboxyl, hydroxyl, aldehyde, ketone, tosyl, silanol, chlorine, hydrazine, hydrazide, photoreactive groups, and any combinations thereof.

83. The detection reagent of any of paragraphs 72-82, wherein said linker is multivalent.

84. The detection reagent of paragraph 83, wherein when the multivalent linker is an avidin-like molecule, both the probe reagent and the nucleic acid label are biotinylated.

85. The detection reagent of any of paragraphs 70-84, wherein said at least one probe reagent is selected from the group consisting of a nucleic acid, an antibody or a portion thereof, an antibody-like molecule, an enzyme, a cell, an antigen, a small molecule, a protein, a peptide, a peptidomimetic, a sugar, a carbohydrate, a lipid, a glycan, a glycoprotein, an aptamer, and any combinations thereof.

86. The detection reagent of any of paragraphs 70-85, wherein said at least one probe reagent is modified.

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87. The detection reagent of any of paragraphs 70-86, wherein said at least one probe reagent is biotinylated.

88. The detection reagent of any of paragraphs 70-87, wherein said at least one nucleic acid label is single-stranded, double-stranded, partially double-stranded, a hairpin, linear, circular, branched, a concatemer, or any combinations thereof.

89. The detection reagent of any of paragraphs 70-88, wherein said at least one nucleic acid label is modified.

90. The detection reagent of any of paragraphs 70-89, wherein said at least one nucleic acid label is designed for minimal cross-hybridization of bases with each other.

91. The detection reagent of any of paragraphs 70-90, wherein said at least one nucleic acid label is conjugated to at least one detectable molecule.

92. The detection reagent of paragraph 91, wherein said at least one detectable molecule is an optical molecule selected from the group consisting of small-molecule dye, a fluorescent protein, a quantum dot, a Raman label, a chromophore, and any combinations thereof.

93. The detection reagent of any of paragraphs 70-92, wherein said at least one nucleic acid label comprises a plurality of pre-determined subsequences.

94. The detection reagent of any of paragraphs 70-93, wherein each of said plurality of predetermined subsequences comprises at least one base.

95. The detection reagent of any of paragraphs 70-94, wherein each of said plurality of predetermined subsequences comprises from 1 to 100 nucleobases.

96. The detection reagent of any of paragraphs 70-95, wherein said plurality of the pre-determined subsequences are conjugated together by at least one sequence linker.

97. The detection reagent of paragraph 96, wherein said sequence linker is a bond.

98. The detection reagent of any of paragraphs 96-97, wherein said sequence linker is a nucleotidic linker.

99. The detection reagent of paragraph 98, wherein said nucleotidic linker is single-stranded, double-stranded, partially double-stranded, a hairpin, or any combinations thereof.

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100. The detection reagent of paragraph 98 or 99, wherein said nucleotidic linker is at least one nucleotide long.

101. The detection reagent of any of paragraphs 70-100, wherein said detection reagent comprises one probe reagent and a plurality of nucleic acid labels.

102. The detecton reagent of any of paragraphs 70-101, wherein said detection reagent comprises a plurality of probe reagents and a nucleic acid label.

103. The detection reagent of any of paragraphs 70-102, wherein said detection reagent comprises a plurality of probe reagents and a plurality of nucleic acid labels.

104. The detection reagent of any of paragraphs 70-103, wherein the detection reagent is adapted for use in immunofluorescence.

105. The detection reagent of any of paragraphs 70-104, wherein the detection reagent is adapted for use in immunohistochemistry.

106. The detection reagent of any of paragraphs 70-105, wherein the detection reagent is adapted for use in fluorescence in situ hybridization.

107. The detection reagent of any of paragraphs 70-106, wherein the detection reagent is adapted for use in western blot.

108. A kit comprising:

a. a plurality of the detection reagents of any of paragraphs 70-107; and
b. at least one reagent.

109. The kit of paragraph 108, wherein the kit further comprises at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.

110. The kit of paragraph 108 or 109, wherein said detection reagents are present in a soluble phase.

111. The kit of any of paragraphs 108-110, wherein said detection reagents are immobilized in a multi-well plate.

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112. The kit of any of paragraphs 108-111, wherein said at least one reagent is selected from the group consisting of a readout reagent, a wash buffer, a signal removal buffer, and any combinations thereof.

113. A kit comprising:

a. a plurality of the nucleic acid labels of the detection reagents of any of paragraphs 70-107;

b. at least one coupling agent that allows a user to conjugate the nucleic acid labels to the user's probe reagents of interest, thereby forming the detection reagents comprising the user's probe reagents of interest; and

c. at least one reagent.

114. The kit of paragraph 113, wherein the kit further comprises at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.

115. The kit of paragraph 113 or 114, wherein the detection reagents comprising the user's probe reagents of interest are present in a soluble phase.

116. The kit of any of paragraphs 113-115, wherein the detection reagents comprising the user's probe reagents of interest are immobilized in a multi-well plate.

117. The kit of any of paragraphs 113-116, wherein said at least one reagent is selected from the group consisting of a readout reagent, a wash buffer, a signal removal buffer, and any combinations thereof.

Some selected definitions

[00291] Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. Unless explicitly stated otherwise, or apparent from context, the terms and phrases below do not exclude the meaning that the term or phrase has acquired in the art to which it pertains. The definitions are provided to aid in describing particular embodiments of the aspects described herein, and are not intended to limit the claimed invention, because the scope of the invention is limited only by the claims. Further, unless

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otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[00292] As used herein the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the invention, yet open to the inclusion of unspecified elements, whether essential or not. Additionally, the term "comprising" or "comprises" includes "consisting essentially of" and "consisting of."

[00293] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of additional elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention.

[00294] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[00295] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about." The term "about" when used in connection with percentages can mean ±1%.

[00296] The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise.

[00297] Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The term "comprises" means "includes." The abbreviation, "e.g." is derived from the Latin exempli gratia, and is used herein to indicate a non-limiting example. Thus, the abbreviation "e.g." is synonymous with the term "for example."

[00298] As used herein, the term "identifier" generally refers to a unique expression to distinguish variations from one to another among a class of substances, items, or objects. In particular embodiments, the term "identifier" as used herein refers to association of a unique pre-determined subsequence to a specific probe reagent, thus conferring the presence and identity of the probe reagent when the pre-determined subsequence is detected.

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[00299] The term "statistically significant" or "significantly" refers to statistical significance and generally means a two standard deviation (2SD) above or below a reference level. The term refers to statistical evidence that there is a difference. It is defined as the probability of making a decision to reject the null hypothesis when the null hypothesis is actually true. The decision is often made using the p-value.

[00300] As used herein, the term "substantially" means a proportion of at least about 60%, or preferably at least about 70% or at least about 80%, or at least about 90%, at least about 95%, at least about 97% or at least about 99% or more, or any integer between 70% and 100%. In some embodiments, the term "substantially" means a proportion of at least about 90%, at least about 95%, at least about 98%, at least about 99% or more, or any integer between 90% and 100%. In some embodiments, the term "substantially" can include 100%.

[00301] The term "sphere" means a particle having an aspect ratio of at most 3:1. The term "aspect ratio" means the ratio of the longest axis of an object to the shortest axis of the object, where the axes are not necessarily perpendicular.

[00302] The term "rod" means a particle having a longest dimension of at most 5000 nm, and having an aspect ratio of from 3:1 to 20:1.

[00303] The term "prism" means a particle having at least two non-parallel faces connected by a common edge.

[00304] As used herein, the term "pharmaceutically acceptable carrier" refers to a pharmaceutically-acceptable material, composition or vehicle for administration of the detection reagents. Pharmaceutically acceptable carriers include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like which are compatible with the activity of the detection reagents and are physiologically acceptable to the subject. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, methylcellulose, ethyl cellulose, microcrystalline cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) lubricating agents, such as magnesium stearate, sodium lauryl sulfate and talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and

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polyethylene glycol (PEG); (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) pH buffered solutions; (21) polyesters, polycarbonates and/or polyanhydrides; (22) bulking agents, such as polypeptides and amino acids (23) serum component, such as serum albumin, HDL and LDL; (22) C2-C12 alchols, such as ethanol; and (23) other non-toxic compatible substances employed in pharmaceutical formulations. Wetting agents, coloring agents, release agents, coating agents, sweetening agents, flavoring agents, perfuming agents, preservative and antioxidants can also be present in the formulation. The terms such as "excipient", "carrier", "pharmaceutically acceptable carrier" are used interchangeably herein.

[00305] As used here, the term "pharmaceutically acceptable" refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[00306] As used herein, the term "therapeutic agent" refers to a biological or chemical agent used for treatment, curing, mitigating, or preventing deleterious conditions in a subject. The term "therapeutic agent" also includes substances and agents for combating a disease, condition, or disorder of a subject, and includes drugs, diagnostics, and instrumentation. "Therapeutic agent" also includes anything used in medical diagnosis, or in restoring, correcting, or modifying physiological functions. The terms "therapeutic agent" and "pharmaceutically active agent" are used interchangeably herein.

[00307] A therapeutic agent can be selected according to the treatment objective and biological action desired. Thus, a therapeutic agent can be selected from any class suitable for the therapeutic objective. Further, the therapeutic agent may be selected or arranged to provide therapeutic activity over a period of time.

[00308] Exemplary pharmaceutically active compound include, but are not limited to, those found in Harrison's Principles of Internal Medicine, 13th Edition, Eds. T.R. Harrison McGraw-Hill N.Y., NY; Physicians Desk Reference, 50th Edition, 1997, Oradell New Jersey, Medical Economics Co.; Pharmacological Basis of Therapeutics, 8th Edition, Goodman and Gilman, 1990; United States Pharmacopeia, The National Formulary, USP XII NF XVII, 1990; current

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edition of Goodman and Oilman's The Pharmacological Basis of Therapeutics; and current edition of The Merck Index, the complete content of all of which are herein incorporated in its entirety.

[00309] Exemplary pharmaceutically active agents include, but are not limited to, steroids and nonsteroidal anti-inflammatory agents, antirestenotic drugs, antimicrobial agents, angiogenic factors, calcium channel blockers, thrombolytic agents, antihypertensive agents, anti-coagulants, antiarrhythmic agents, cardiac glycosides, and the like.

[00310] In some embodiments, the therapeutic agent is selected from the group consisting of salicylic acid and derivatives (aspirin), para-aminophenol and derivatives (acetaminophen), arylpropionic acids (ibuprofen), corticosteroids, histamine receptor antagonists and bradykinin receptor antagonists, leukotriene receptor antagonists, prostaglandin receptor antagonists, platelet activating factor receptor antagonists, sulfonamides, trimethoprim-sulfamethoxazole, quinolones, penicillins, cephalosporin, basic fibroblast growth factor (FGF), acidic fibroblast growth factor, vascular endothelial growth factor, angiogenic transforming growth factor alpha and beta, tumor necrosis factor, angiopoietin, platelet-derived growth factor, dihydropyridines (e.g., nifedipine, benzothiazepines such as dilitazem, and phenylalkylamines such as verapamil), urokinase plasminogen activator, urokinase, streptokinase, angiotensin converting enzyme (ACE) inhibitors, spironolactone, tissue plasminogen activator (tPA), diuretics, thiazides, antiadrenergic agents, clonidine, propanolol, angiotensin-converting enzyme inhibitors, captopril, angiotensin receptor antagonists, losartan, calcium channel antagonists, nifedine, heparin, warfarin, hirudin, tick anti-coagulant peptide, and low molecular weight heparins such as enoxaparin, lidocaine, procainamide, encainide, flecanide, beta adrenergic blockers, propranolol, amiodarone, verpamil, diltiazem, nickel chloride, cardiac glycosides, angiotensin converting enzyme inhibitors, angiotensin receptor antagonists, nitrovasodilators, hypolipidemic agents (e.g., nicotinic acid, probucol, etc.), bile acid-binding resins (e.g., cholestyramine, and fibric acid derivatives e.g., clofibrate), HMG CoA reductase inhibitors, HMG CoA synthase inhibitors, squalene synthase inhibitors, squalene epoxidase inhibitors, statins (e.g., lovastatin, cerivastatin, fluvastatin, pravastatin, simvaststin, etc.), anti-psychotics, SSRIs, antiseizure medication, contraceptives, systemic and local analgesics (chronic pain, bone growth/remodeling factors (osteoblast/osteoclast recruiting and stimulating factors), neurotransmitters (L-DOPA, Dopamine, neuropeptides), emphysema drugs, TGF-beta), rapamycin, naloxone, paclitaxel,

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amphotericin, Dexamethasone, flutamide, vancomycin, phenobarbital, cimetidine, atenolol, aminoglycosides, hormones (e.g., thyrotropin-releasing hormone, p-nitrophenyl beta-cellopentaosideand luteinizing hormone-releasing hormone), vincristine, amiloride, digoxin, morphine, procainamide, quinidine, quinine, ranitidine, triamterene, trimethoprim, vancomycin, aminoglycosides, and penicillin, and pharmaceutically acceptable salts thereof.

[00311] As used herein, the term "fluorescent color" refers to a color emitted by any fluorophore (e.g., fluorescent dye, fluorescent particles such as quantum dots, and/or fluorescent proteins) upon light excitation and/or electromagnetic radiation. Fluorescent dye as disclosed herein refers to a dye which exhibits energy and is visible under illumination at a predefined wavelength. Numerous fluorescent molecules are commercially available and can be adapted for use in the methods, detection reagents and kits as disclosed herein, and include those from Molecular Probes, Sigma and similar other commercial sources. In some embodiments, a "fluorescent color" can be produced by phosphorescence. In some embodiments, a "fluorescent color" can be produced by luminescence (including bioluminescence).

[00312] To the extent not already indicated, it will be understood by those of ordinary skill in the art that any one of the various embodiments herein described and illustrated may be further modified to incorporate features shown in any of the other embodiments disclosed herein.

[00313] The following examples illustrate some embodiments and aspects of the invention. It will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be performed without altering the spirit or scope of the invention, and such modifications and variations are encompassed within the scope of the invention as defined in the claims which follow. The following examples do not in any way limit the invention.

EXAMPLES

Example 1: Exemplary hybridization-based detection methods of detection reagents

[00314] A solution suspension of streptavidin-coated Dynabeads (of about 1 μm), each conjugated to one of six nucleic acid labels and a corresponding probe reagent specific for a target analyte, wherein each of the nucleic acid labels comprised at least one pre-determined subsequence, e.g., a first (e.g., A1, A2, and A3) and a second (B1, B2, and B3) pre-determined subsequence, was prepared. The nucleic acid labels can comprise a nucleic acid sequence of any

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length. In some embodiments, the nucleic acid labels can comprise at least 15 nucleotides, at least 20 nucleotides, at least 22 nucleotides, or at least 24 nucleotides. A sample containing multiple target analytes was contacted with the target analyte-specific Dynabeads, so that the Dynabeads would bind to the respective target analyte on the sample. Any unbound Dynabeads was then removed, e.g., by washing. In the first readout, a set of three decoder probes, each with a distinct sequence complementary to the first pre-determined subsequences (e.g., A1*, A2*, and A3*) and a corresponding optical label (e.g., selected from red, green or blank), was added to the sample with bound Dynabeads, and then imaged with fluorescent microscopy (**Fig. 6A**). The fluorescent signal was then removed, e.g., by photobleaching, from the previous stage before the next readout. In the second readout, a different set of three decoder probes, each with a distinct complementary sequence to the second pre-determined subsequences (e.g., B1*, B2*, B3*) and a corresponding optical label (e.g., selected from red, green blank), was added to the photobleached sample, and then imaged with fluorescent microscopy (**Fig. 6B**). The temporal sequence of the optical signatures obtained from each bead can then be analyzed to determine the identity of the probe and thus the presence of the corresponding target analyte on the sample. Based on the intensity and/or coverage of the optical signals, the amount of the target analyte can also be determined.

[00315] By way of example only, a biotinylated anti-*C. albicans* antibody (e.g., an commercially-available antibody from Pierce Antibodies PA1-27145) was conjugated with a plurality of different biotinylated nucleic acid labels (e.g., 8 different biotinylated SeqTag label sequences) using any conjugation methods known in the art. In one embodiment, the biotinylated anti-*C. albicans* antibody was conjugated with a plurality of different biotinylated nucleic acid labels (e.g., biotinylated SeqTag label sequences) using a streptavidin-like protein (e.g., Neutravidin) as a bridge. The SeqTag label sequences are shown in Table 5. The SeqTag label sequences can comprise at least about 24 nucleotides. In some embodiments, the SeqTag label sequences can comprise DNA, RNA or a combination thereof. Each conjugate was incubated with a sample comprising *C. albicans*, washed, and readout using any readout method as described herein, e.g., the displacement-hybridization method.

Table 5. DNA sequences of exemplary SeqTag labels

SEQ ID NO:	SeqTag label	DNA Sequence

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59	SeqTag 1	5'-Biotin – TTTCGCTTTCTGTAATGGAGTGGA – 3'
60	SeqTag 2	5'-Biotin – TTTCGCTTTAGCCTAAGTGAAATC – 3'
61	SeqTag 3	5'-Biotin – TTTCGCTTTTTTGGGGAAAAGACA – 3'
62	SeqTag 4	5'-Biotin – TTTCGCTTTTAGGCATTAGCATTG – 3'
63	SeqTag 5	5'-Biotin – TTTCGCTTTGGAAGCACCTATTCC – 3'
64	SeqTag 6	5'-Biotin – TTTCGCTTTCTGGAGAAAGGGCCA – 3'
65	SeqTag 7	5'-Biotin – TTTCGCTTTCGGTTCCAAAGACAC – 3'
66	SeqTag 8	5'-Biotin – TTTCGCTTTGAAGCCGGTTATAGC – 3'

[00316] During the displacement hybridization method, by way of example only, as shown in **Fig. 7**, the yeast was incubated in readout step 1 with a mixture of all three "set 1" decoder probes, followed by an incubation with a mixture of the "set 2" decoder probes and the "set 1 displacers" (which are nucleic acid sequences to remove "set 1" fluorescence), and similarly with appropriate sets of decoder probes and displacers for steps 3 and 4. The decoder probes can comprise a nucleic acid sequence (e.g., DNA, RNA or a combination thereof) and a detection label, e.g., at its 5' end. The nucleic acid sequence of the decoder probes can be of any length. In some embodiments, the decoder probes can comprise at least about 10 nucleotides, at least about 12 nucleotides, at least about 14 nucleotides, at least about 16 nucleotides, at least about 18 nucleotides, at least about 20 nucleotides or longer. Detection labels can be any art-recognized label described herein, e.g., fluorescent detection labels such as FAM, Cy3, and C5, or any labels described herein. Probe displacers, as described earlier, are nucleic acid sequences that are used to remove or displace the previous decoder probes hybridized to the nucleic acid labels (e.g., SeqTag labels) such that a different set of decoder probes can be added and hybridized to the nucleic acid labels. The probe displacers can have a nucleic acid sequence of any length. In some embodiments, the nucleic acid sequence of the probe displacers can be longer than that of the decoder probes. In some embodiments, the probe displacers can comprise at least about 12 nucleotides, at least about 14 nucleotides, at least about 16 nucleotides, at least about 18 nucleotides, at least about 20 nucleotides, at least about 21 nucleotides or longer. Tables 2 and 3 show DNA sequences of exemplary decoder probes (or readout probes) and probe displacers, respectively. As seen in **Fig. 7**, each biotinylated anti-*C albicans* antibody conjugated with a different SeqTag label sequence properly stained the yeast and fluorescenced only as designated by its SeqTag label with appropriate sets of decoder probes and probe displacer in each readout step.

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Table 6. DNA sequences of exemplary decoder probes

SEQ ID NO:	Readout probe	DNA Sequence
67	Set 1 FAM	5'-FAM – TTTTCCACTCCATTACAG – 3'
68	Set 1 Cy3	5'-Cy3 – TTTGATTTCACTTAGGCT – 3'
69	Set 1 Cy5	5'-Cy5 – TTTTGTCTTTTCCCCAAA – 3'
70	Set 2 FAM	5'-FAM – TTTCAATGCTAATGCCTA – 3'
71	Set 2 Cy3	5'-Cy3 – TTTGGAATAGGTGCTTCC – 3'
72	Set 2 Cy5	5'-Cy5 – TTTTGGCCCTTTCTCCAG – 3'
73	Set 3 FAM	5'-FAM – TTTGTGTCTTTGGAACCG – 3'
74	Set 3 Cy3	5'-Cy3 – TTTGCTATAACCGGCTTC – 3'

Table 7. DNA sequences of exemplary probe displacers

SEQ ID NO:	Probe Displacer	DNA Sequence
75	Set 1 FAM-Displacer	5' – TCCACTCCATTACAGAAAGCG – 3'
76	Set 1 Cy3-Displacer	5' – GATTTCACTTAGGCTAAAGCG – 3'
77	Set 1 Cy5-Displacer	5' – TGTCTTTTCCCCAAAAAAGCG – 3'
78	Set 2 FAM-Displacer	5' – CAATGCTAATGCCTAAAAGCG – 3'
79	Set 2 Cy3-Displacer	5' – GGAATAGGTGCTTCCAAAGCG – 3'
80	Set 2Cy5-Displacer	5' – TGGCCCTTTCTCCAGAAAGCG – 3'
81	Set 3 FAM-Displacer	5' –TGTGTCTTTGGAACCGAAAGCG – 3'
82	Set 3 Cy3-Displacer	5' – GCTATAACCGGCTTCAAAGCG – 3'

[00317] Content of all patents and other publications identified herein is expressly incorporated herein by reference for all purposes. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

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CLAIMS

What is claimed is:

1. A method for detecting a plurality of analytes in a sample, comprising:

    a. contacting the sample with a composition comprising a plurality of detection reagents, wherein each subpopulation of the detection reagents targets at least one different analyte, and wherein each detection reagent comprises:

        at least one probe reagent targeting an analyte and at least one nucleic acid label comprising one or a plurality of pre-determined subsequences, wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together; and wherein at least a portion of said one or the plurality of pre-determined subsequences form an identifier of said at least one probe reagent; and

    b. detecting in a temporally-sequential manner said one or the plurality of the pre-determined subsequences of said detection reagent, wherein said detection of the subsequences each generates a signal signature corresponding to said subsequence, and wherein a temporal order of the signal signatures corresponding to said one or the plurality of the subsequences of said detection reagent identifies a subpopulation of the detection reagents.

2. The method of claim 1, wherein said each subpopulation of the detection reagents targets a set of analytes.

3. The method of claim 1 or 2, wherein the temporal order of the signal signatures corresponding to said one or the plurality of the subsequences of said detection reagent is unique for each subpopulation of the detection reagents.

4. The method of any of claims 1-3, wherein said detection reagents are present in a soluble phase.

5. The method of any of claims 1-4, further comprising processing said sample before said contacting with said plurality of detection reagents.

6. The method of any of claims 1-5, further comprising removing any unbound detection reagents before the detecting step (b).

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7. The method of any of claims 1-6, further comprising comparing said temporal order of the signal signatures with different identifiers of said at least one probe reagent, wherein an agreement between the temporal order of the signal signatures and a particular identifier of said at least one probe reagent identifies the analyte in the sample.

8. The method of any of claims 1-7, further comprising measuring the intensity of the signal signatures generated from each subpopulation of the detection reagents.

9. The method of claim 8, wherein the intensity of the signal signatures generated from each subpopulation of the detection reagents indicates an amount of the analyte.

10. The method of claim 8 or 9, wherein the intensity of the signal signatures generated from each subpopulation of the detection reagents is used in identification of the subpopulation of the detection reagents.

11. The method of any of claims 1-10, wherein said detecting of step (b) comprises sequencing.

12. The method of claim 11, wherein said sequencing is performed via ligation, hybridization, synthesis, amplification, or single-base extension.

13. The method of any of claims 1-12 wherein said detecting of step (b) comprises hybridizing a decoder probe with said subsequence, wherein said decoder probe comprises a detectable label.

14. The method of any of claims 1-13, wherein said detecting of step (b) comprises:

    a. hybridizing a set of decoder probes with a subsequence of the detection reagents, wherein each subpopulation of the decoder probes comprises a detectable label, each detectable label producing a signal signature;

    b. detecting said signal signature produced by the hybridization of said set of decoder probes;

    c. optionally removing said different signal signature produced by the hybridization of said set of decoder probes; and

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d. repeating steps (a) through (c) for other subsequences of said detection reagents, thereby producing a temporal order of the signal signatures corresponding to said each detection reagent.

15. The method of claim 14, wherein said each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.

16. The method of claim 14 or 15, wherein said each subpopulation of the decoder probes is at least partially or completely complementary to said subsequence of the detection reagents.

17. The method of any of claims 14-16, wherein at least two or more subpopulations of the decoder probes are at least partially or completely complementary to the same subsequence of the detection reagents.

18. The method of any of claims 14-17, wherein said removing step is performed by washing, heating, photobleaching, displacement, cleavage, enzymatic digestion, quenching, chemical degradation, bleaching, oxidation or any combinations thereof.

19. The method of any of claims 14-18, wherein said detectable label comprises or is an optical label selected from the group consisting of a small-molecule dye, a fluorescent molecule, a fluorescent protein, a quantum dot, Raman label, a chromophore, and any combinations thereof.

20. The method of any of claims 14-19, wherein said detectable label comprises or is a colorimetric reagent.

21. The method of any of claims 14-20, wherein said detectable label comprises or is a Raman label.

22. The method of any of claims 1-21, wherein said signal signatures comprise or are optical signatures.

23. The method of claim 22, wherein said optical signatures comprise signatures of fluorescent color, visible light, no-color, Raman label, or any combinations thereof.

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24. The method of claim 22, wherein said optical signatures comprise signatures of one or more fluorescent colors, one or more visible lights, one or more no-colors, one or more Raman labels, or any combinations thereof.

25. The method of any of claims 22-24, wherein said optical signatures are detected by optical imaging or spectroscopy.

26. The method of any of claims 1-25, wherein said analytes are selected from the group consisting of antigens, receptors, proteins, peptides, sugars, glycoproteins, peptidoglycans, lipids, nucleic acids, oligonucleotides, cells, viruses, and any combinations thereof.

27. The method of any of claims 1-26, wherein said nucleic acids are selected from the group consisting of cellular DNA or RNA, messenger RNA, microRNA, ribosomal RNA, and any combinations thereof.

28. The method of any of claims 1-27, wherein said sample is a protein sample immobilized on a solid support.

29. The method of claim 28, wherein the solid support is a blotting membrane.

30. The method of any of claims 1-27, wherein said sample is a biological sample.

31. The method of claim 30, wherein said biological sample comprises one or more cells, one or more tissues, one or more fluids or any combinations thereof.

32. The method of claim 30 or 31, wherein said biological sample comprises blood, sputum, cerebrospinal fluid, urine, saliva, sperm, sweat, mucus, nasal discharge, vaginal fluids or any combinations thereof.

33. The method of any of claims 30-31, wherein the said biological sample comprises a biopsy, a surgically removed tissue, a swap or any combinations thereof.

34. The method of claims 1-27, wherein said sample comprises an environmental sample, food, food byproduct, soil, an archaeological sample, an extraterrestrial sample, or any combinations thereof.

35. The method of any of claims 1-34, wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together by at least one linker.

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36. The method of claim 35, wherein said linker is a bond.

37. The method of claim 35 or 36, wherein said linker is a linker molecule.

38. The method of claim 37, wherein said linker molecule is a polymer, sugar, nucleic acid, peptide, protein, hydrocarbon, lipid, polyethylene glycol, crosslinker, or any combinations thereof.

39. The method of any of claims 35-38, wherein said linker is a particle.

40. The method of claim 39, wherein said particle is selected from a group consisting of a gold nanoparticle, a magnetic bead or nanoparticle, a polystyrene bead, a nanotube, a nanowire, a microparticle, and any combinations thereof.

41. The method of claim 40, wherein said particle is a nanoparticle.

42. The method of any of claims 39-41, wherein said particle is modified.

43. The method of any of claims 39-42, wherein said particle is coated with streptavidin or a derivative thereof.

44. The method of any of claims 39-43, wherein said particle is modified with at least one functional group.

45. The method of claim 44, wherein said at least one functional group is selected from the group consisting of amine, carboxyl, hydroxyl, aldehyde, ketone, tosyl, silanol, chlorine, hydrazine, hydrazide, photoreactive groups, and any combinations thereof.

46. The method of any of claims 35-45, wherein said linker is multivalent.

47. The method of claim 46, wherein when the multivalent linker is an avidin-like molecule, both the probe reagent and the nucleic acid label are biotinylated.

48. The method of any of claims 1-47, wherein said at least one probe reagent is selected from the group consisting of a nucleic acid, an antibody or a portion thereof, an antibody-like molecule, an enzyme, a cell, an antigen, a small molecule, a protein, a peptide, a peptidomimetic, a sugar, a carbohydrate, a lipid, a glycan, a glycoprotein, an aptamer, and any combinations thereof.

49. The method of any of claims 1-48, wherein said at least one probe reagent is modified.

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50. The method of any of claims 1-49, wherein said at least one probe reagent is biotinylated.

51. The method of any of claims 1-50, wherein said at least one nucleic acid label is single-stranded, double-stranded, partially double-stranded, a hairpin, linear, circular, branched, a concatemer, or any combinations thereof.

52. The method of any of claims 1-51, wherein said at least one nucleic acid label is modified.

53. The method of any of claims 1-52, wherein said at least one nucleic acid label is designed for minimal cross-hybridization of bases with each other.

54. The method of any of claims 1-53, wherein said at least one nucleic acid label is conjugated to at least one detectable molecule.

55. The method of claim 54, wherein said at least one detectable molecule is an optical molecule selected from the group consisting of a small-molecule dye, a fluorescent protein, a quantum dot, a Raman label, a chromophore, and any combinations thereof.

56. The method of any of claims 1-55, wherein each of said plurality of the pre-determined subsequences comprises at least one base.

57. The method of any of claims 1-56, wherein each of said plurality of the pre-determined subsequences comprises from 1 to 100 nucleobases.

58. The method of any of claims 1-57, wherein said plurality of the pre-determined subsequences are conjugated together by at least one sequence linker.

59. The method of claim 58, wherein said sequence linker is a bond.

60. The method of any of claims 58-59, wherein said sequence linker is a nucleotidic linker.

61. The method of claim 60, wherein said nucleotidic linker is single-stranded, double-stranded, partially double-stranded, a hairpin or any combinations thereof.

62. The method of claim 60 or 61, wherein said nucleotidic linker is at least one nucleotide long.

63. The method of any of claims 1-62, wherein said detection reagent comprises one probe reagent and a plurality of nucleic acid labels.

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64. The method of any of claims 1-62, wherein said detection reagent comprises a plurality of probe reagents and a nucleic acid label.

65. The method of any of claims 1-62, wherein said detection reagent comprises a plurality of probe reagents and a plurality of nucleic acid labels.

66. The method of any of claims 1-65, wherein the method is adapted for use in immunofluorescence.

67. The method of any of claims 1-66, wherein the method is adapted for use in immunohistochemistry.

68. The method of any of claims 1-67, wherein the method is adapted for use in fluorescence in situ hybridization.

69. The method of any of claims 1-68, wherein the method is adapted for use in western blot.

70. A detection reagent comprising at least one probe reagent and at least one nucleic acid label,

wherein said at least one nucleic acid label comprises at least one pre-determined subsequence to be detected in a temporally-sequential manner;

wherein said at least one pre-determined subsequence forms an identifier of said at least one probe reagent; and

wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together.

71. The detection reagent of claim 70, wherein the detection reagent is present in a soluble phase.

72. The detection reagent of claim 70 or 71, wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together by at least one linker.

73. The detection reagent of claim 72, wherein said linker is a bond.

74. The detection reagent of claim 72 or 73, wherein said linker is a linker molecule.

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75. The detection reagent of claim 74, wherein said linker molecule is a polymer, sugar, nucleic acid, peptide, protein, hydrocarbon, lipid, polyethelyne glycol, crosslinker or combination thereof.

76. The detection reagent of any of claims 72-75, wherein said linker is a particle.

77. The detection reagent of claim 76, wherein said particle is selected from a group consisting of a gold nanoparticle, a magnetic bead or nanoparticle, a polystyrene bead, a nanotube, a nanowire, a microparticle, and any combinations thereof.

78. The detection reagent of claim 76 or 77, wherein said particle is a nanoparticle.

79. The detection reagent of any of claims 76-78, wherein said particle is modified.

80. The detection reagent of any of claims 76-79, wherein said particle is coated with streptavidin or a derivative thereof.

81. The detection reagent of any of claims 76-80, wherein said particle is modified with at least one functional group.

82. The detection reagent of claim 81, wherein the at least one functional group is selected from the group consisting of amine, carboxyl, hydroxyl, aldehyde, ketone, tosyl, silanol, chlorine, hydrazine, hydrazide, photoreactive groups, and any combinations thereof.

83. The detection reagent of any of claims 72-82, wherein said linker is multivalent.

84. The detection reagent of claim 83, wherein when the multivalent linker is an avidin-like molecule, both the probe reagent and the nucleic acid label are biotinylated.

85. The detection reagent of any of claims 70-84, wherein said at least one probe reagent is selected from the group consisting of a nucleic acid, an antibody or a portion thereof, an antibody-like molecule, an enzyme, a cell, an antigen, a small molecule, a protein, a peptide, a peptidomimetic, a sugar, a carbohydrate, a lipid, a glycan, a glycoprotein, an aptamer, and any combinations thereof.

86. The detection reagent of any of claims 70-85, wherein said at least one probe reagent is modified.

87. The detection reagent of any of claims 70-86, wherein said at least one probe reagent is biotinylated.

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88. The detection reagent of any of claims 70-87, wherein said at least one nucleic acid label is single-stranded, double-stranded, partially double-stranded, a hairpin, linear, circular, branched, a concatemer, or any combinations thereof.

89. The detection reagent of any of claims 70-88, wherein said at least one nucleic acid label is modified.

90. The detection reagent of any of claims 70-89, wherein said at least one nucleic acid label is designed for minimal cross-hybridization of bases with each other.

91. The detection reagent of any of claims 70-90, wherein said at least one nucleic acid label is conjugated to at least one detectable molecule.

92. The detection reagent of claim 91, wherein said at least one detectable molecule is an optical molecule selected from the group consisting of small-molecule dye, a fluorescent protein, a quantum dot, a Raman label, a chromophore, and any combinations thereof.

93. The detection reagent of any of claims 70-92, wherein said at least one nucleic acid label comprises a plurality of pre-determined subsequences.

94. The detection reagent of any of claims 70-93, wherein each of said plurality of predetermined subsequences comprises at least one base.

95. The detection reagent of any of claims 70-94, wherein each of said plurality of predetermined subsequences comprises from 1 to 100 nucleobases.

96. The detection reagent of any of claims 70-95, wherein said plurality of the pre-determined subsequences are conjugated together by at least one sequence linker.

97. The detection reagent of claim 96, wherein said sequence linker is a bond.

98. The detection reagent of any of claims 96-97, wherein said sequence linker is a nucleotidic linker.

99. The detection reagent of claim 98, wherein said nucleotidic linker is single-stranded, double-stranded, partially double-stranded, a hairpin, or any combinations thereof.

100. The detection reagent of claim 98 or 99, wherein said nucleotidic linker is at least one nucleotide long.

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101. The detection reagent of any of claims 70-100, wherein said detection reagent comprises one probe reagent and a plurality of nucleic acid labels.

102. The detecton reagent of any of claims 70-101, wherein said detection reagent comprises a plurality of probe reagents and a nucleic acid label.

103. The detection reagent of any of claims 70-102, wherein said detection reagent comprises a plurality of probe reagents and a plurality of nucleic acid labels.

104. The detection reagent of any of claims 70-103, wherein the detection reagent is adapted for use in immunofluorescence.

105. The detection reagent of any of claims 70-104, wherein the detection reagent is adapted for use in immunohistochemistry.

106. The detection reagent of any of claims 70-105, wherein the detection reagent is adapted for use in fluorescence in situ hybridization.

107. The detection reagent of any of claims 70-106, wherein the detection reagent is adapted for use in western blot.

108. A kit comprising:

a. a plurality of the detection reagents of any of claims 70-107; and
b. at least one reagent.

109. The kit of claim 108, wherein the kit further comprises at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.

110. The kit of claim 108 or 109, wherein said detection reagents are present in a soluble phase.

111. The kit of any of claims 108-110, wherein said detection reagents are immobilized in a multi-well plate.

112. The kit of any of claims 108-111, wherein said at least one reagent is selected from the group consisting of a readout reagent, a wash buffer, a signal removal buffer, and any combinations thereof.

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113. A kit comprising:

a. a plurality of the nucleic acid labels of the detection reagents of any of claims 70-107;

b. at least one coupling agent that allows a user to conjugate the nucleic acid labels to the user's probe reagents of interest, thereby forming the detection reagents comprising the user's probe reagents of interest; and

c. at least one reagent.

114. The kit of claim 113, wherein the kit further comprises at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.

115. The kit of claim 113 or 114, wherein the detection reagents comprising the user's probe reagents of interest are present in a soluble phase.

116. The kit of any of claims 113-115, wherein the detection reagents comprising the user's probe reagents of interest are immobilized in a multi-well plate.

117. The kit of any of claims 113-116, wherein said at least one reagent is selected from the group consisting of a readout reagent, a wash buffer, a signal removal buffer, and any combinations thereof.

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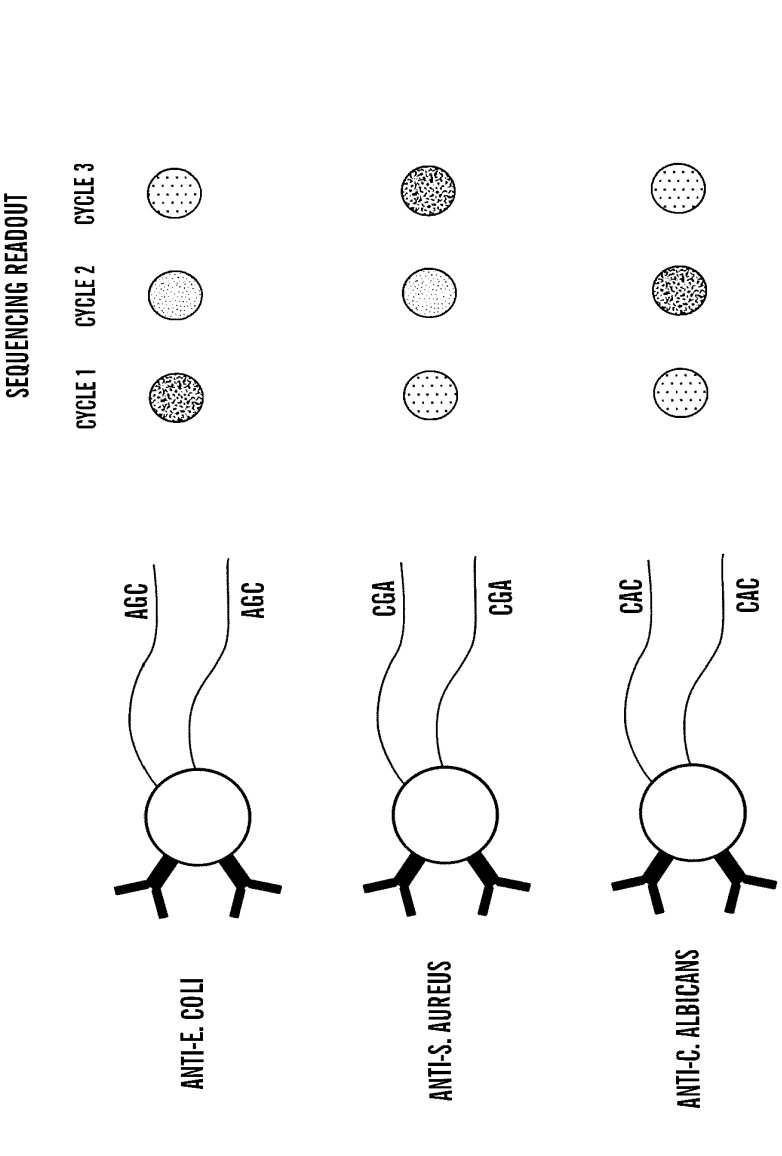


FIG. 1

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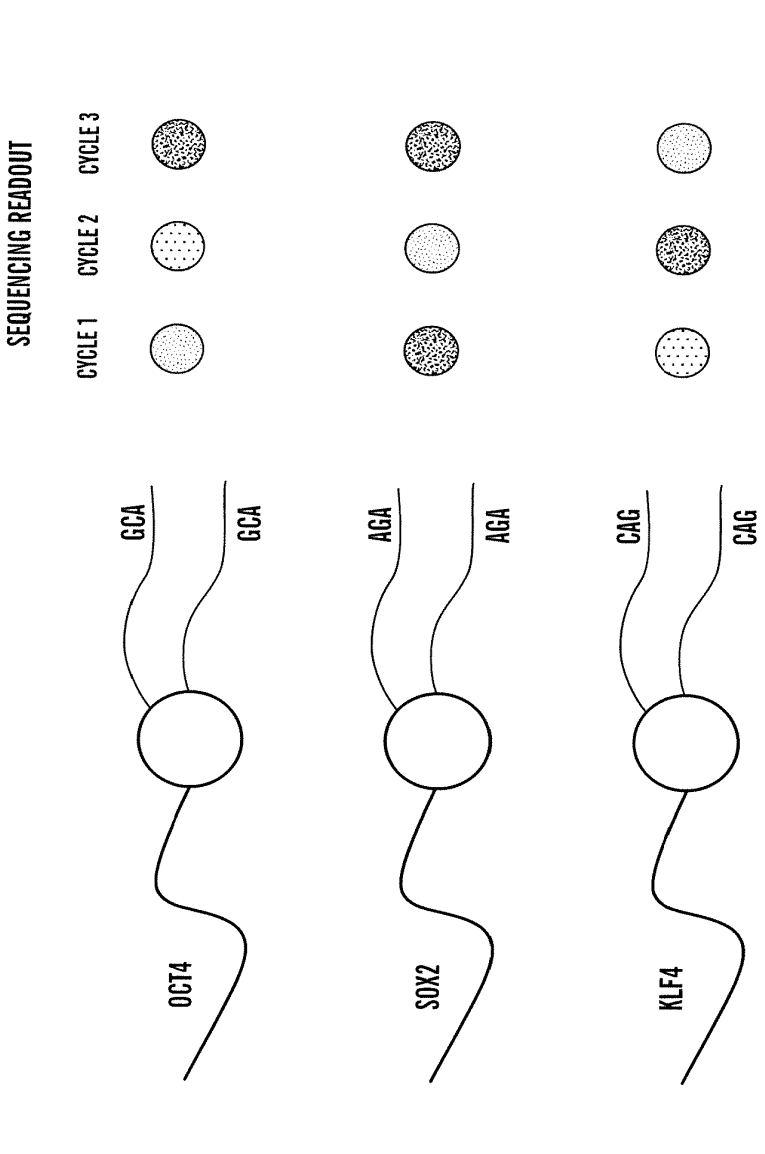


FIG. 2

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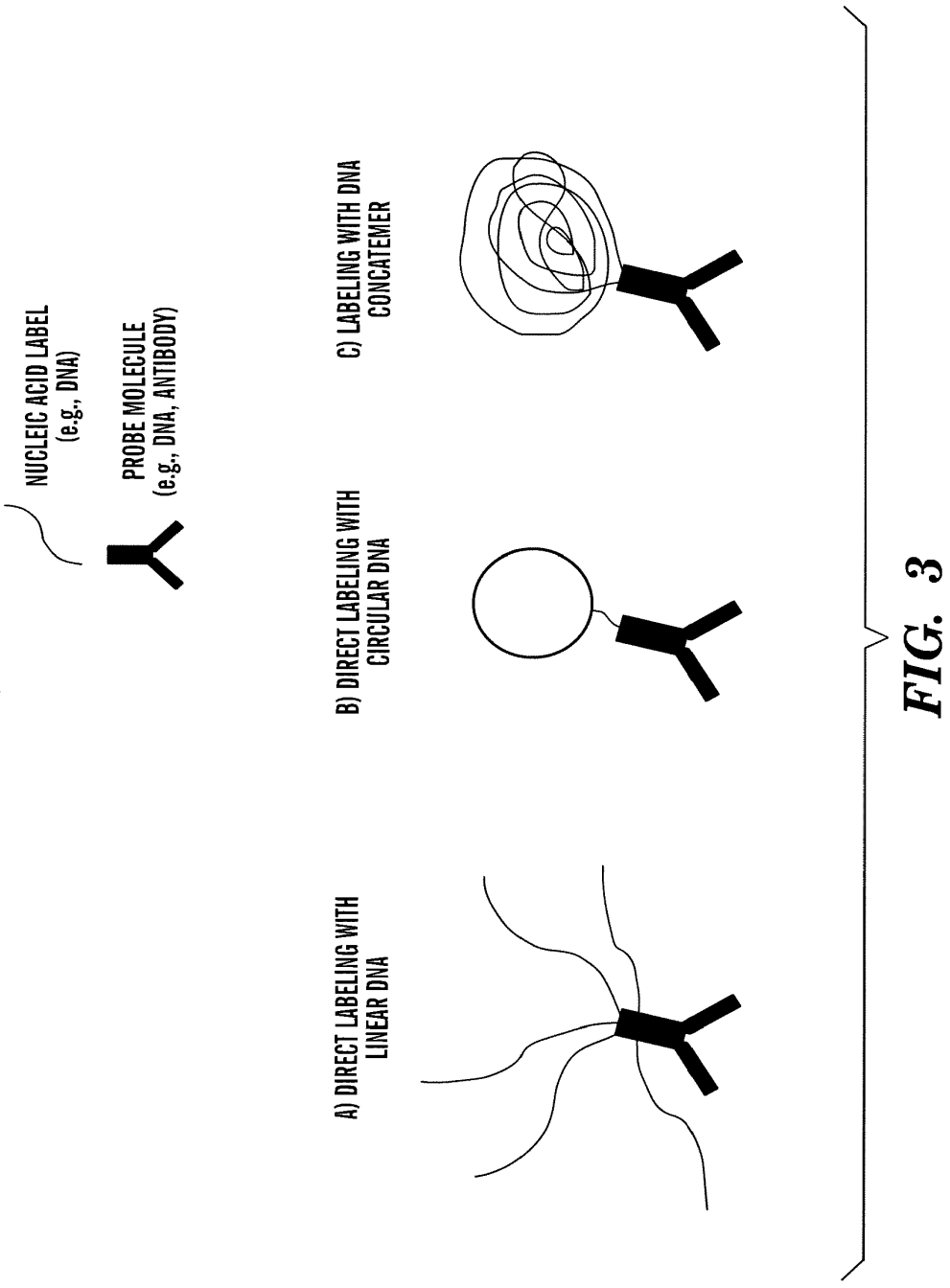


FIG. 3

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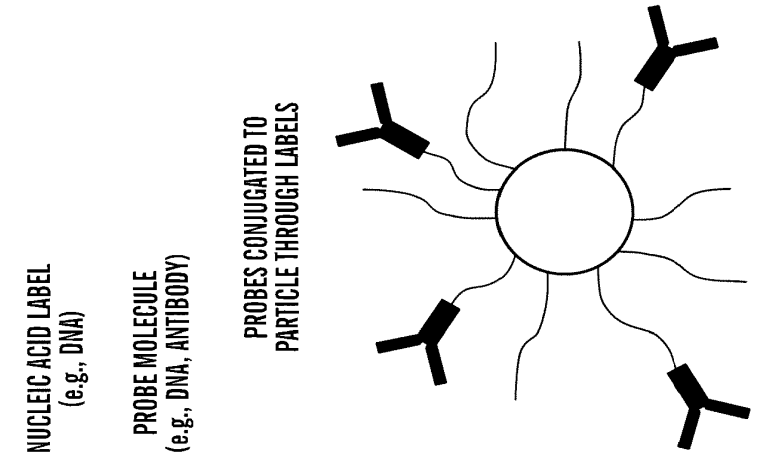


FIG. 4B

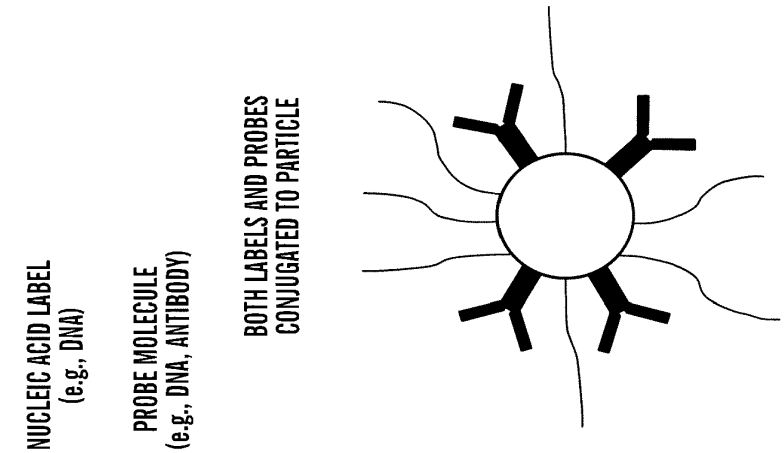


FIG. 4A

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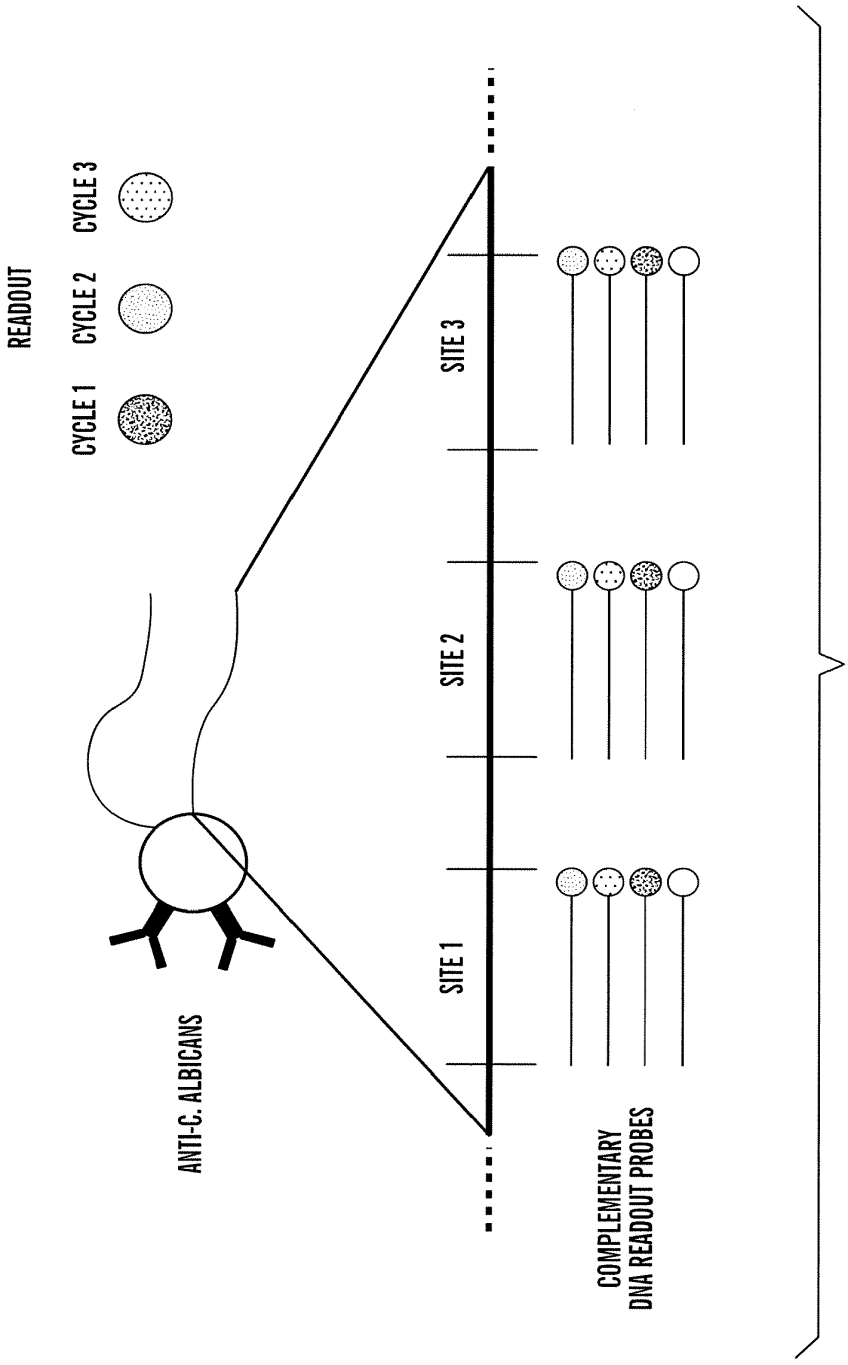


FIG. 5

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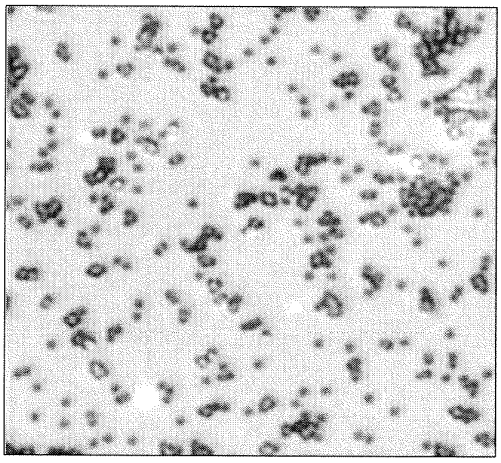


FIG. 6B

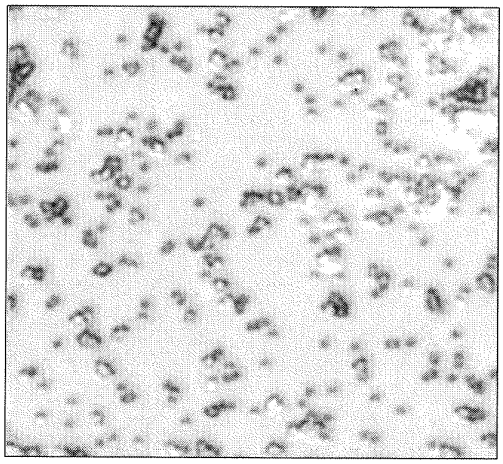


FIG. 6A

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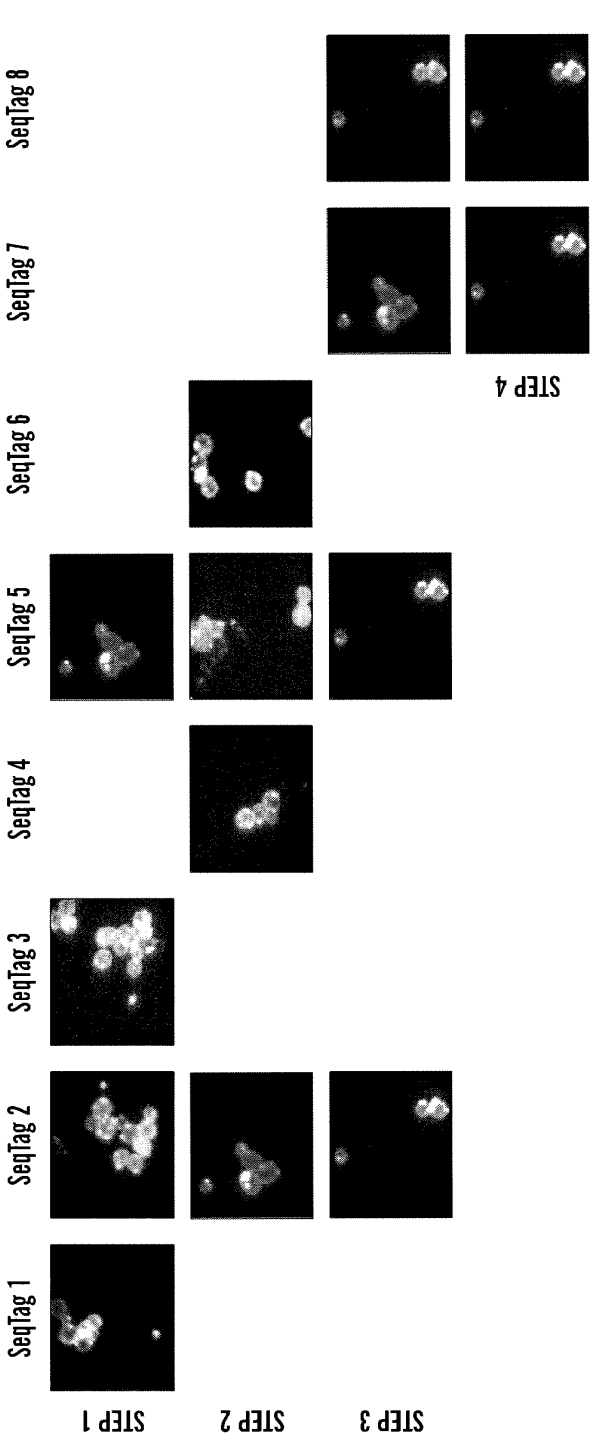


FIG. 7

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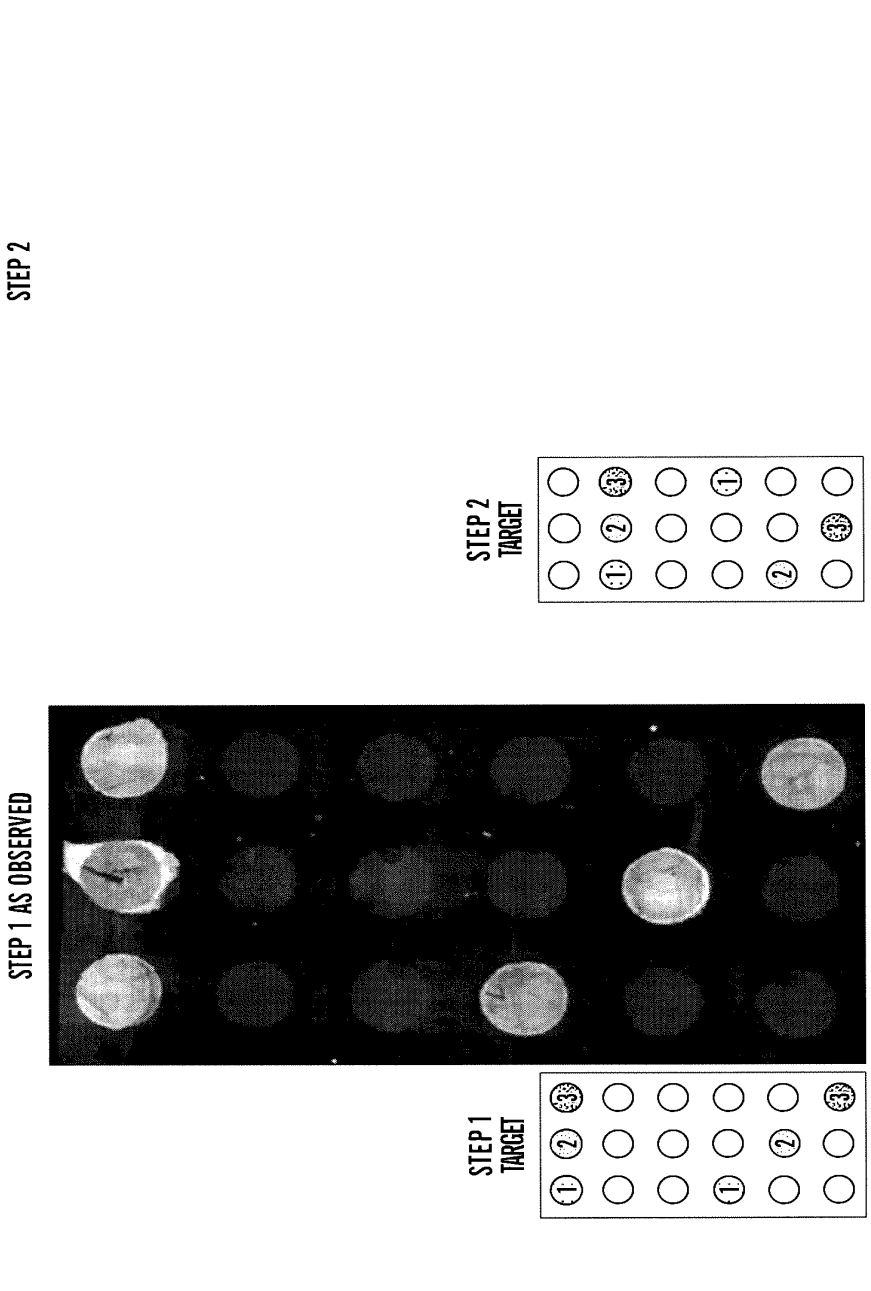


FIG. 8

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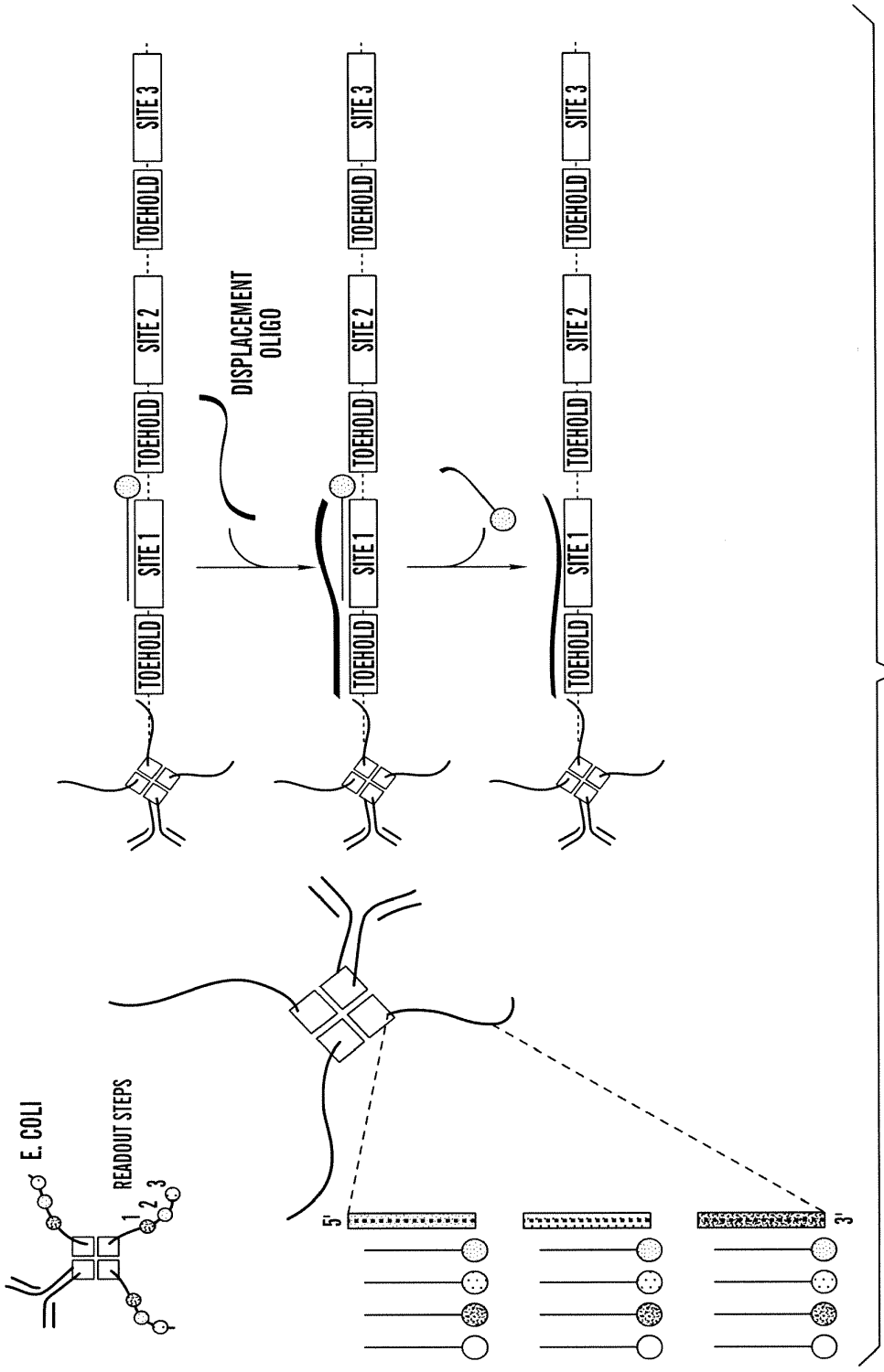


FIG. 9

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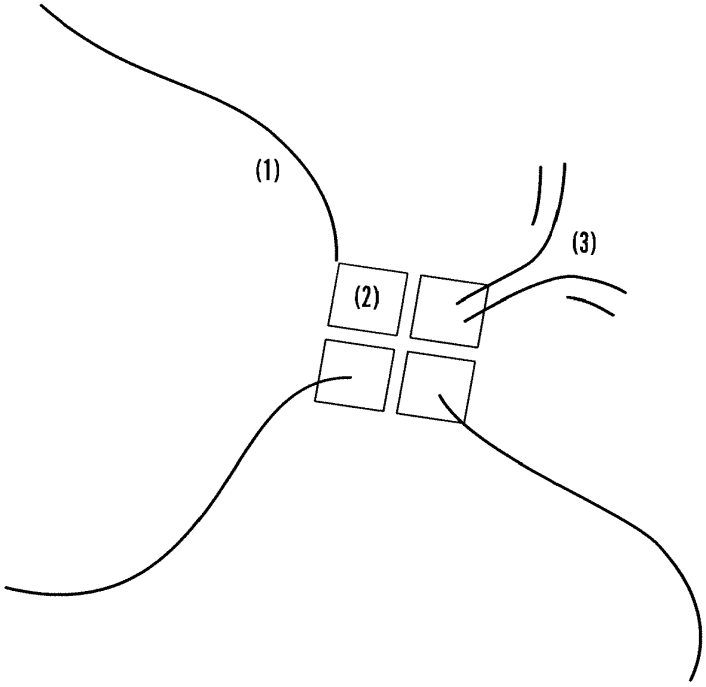


FIG. 10

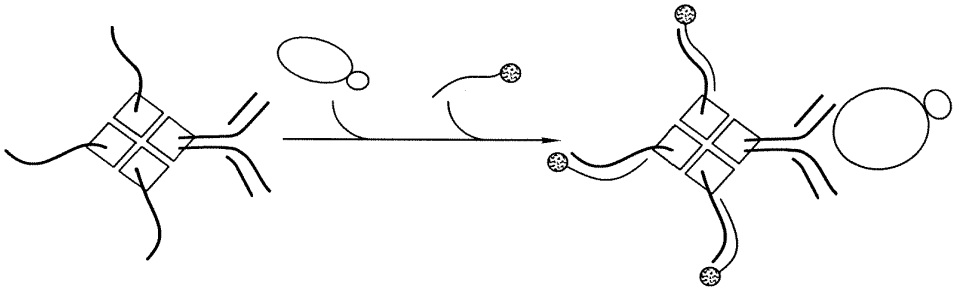


FIG. 11

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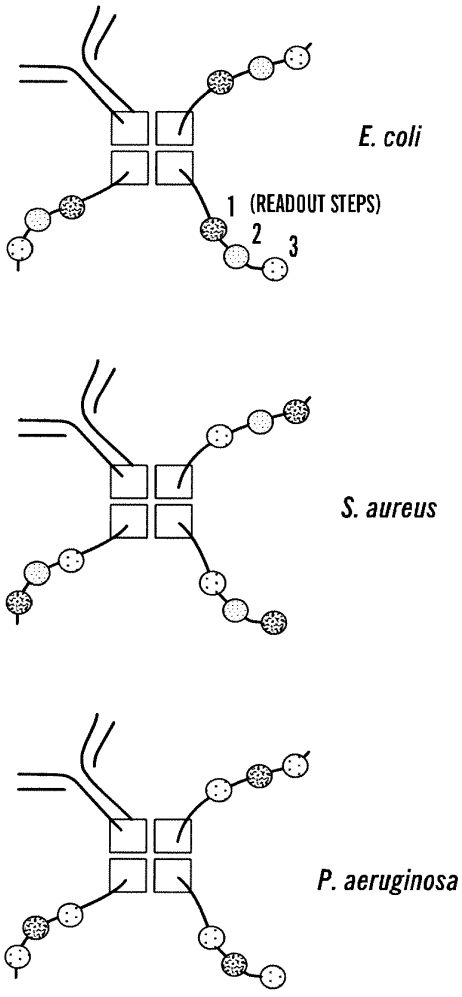


FIG. 12

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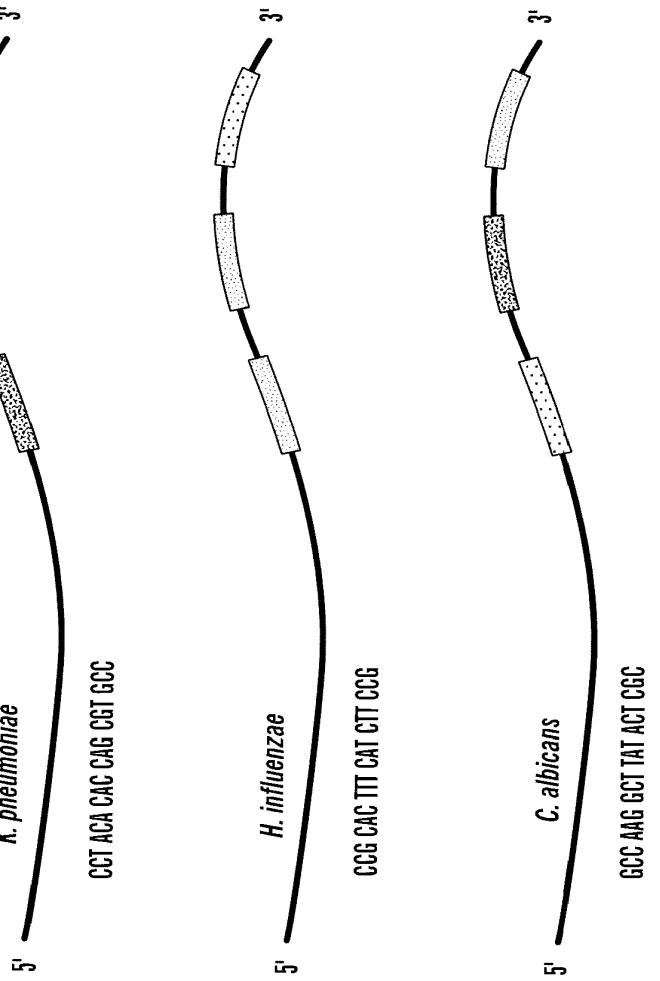


FIG. 13

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/071398

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/68(2006.01)i, C12N 15/11(2006.01)i, G01N 33/68(2006.01)i, G01N 33/532(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q 1/68; C07K 19/00; G01N 33/48; G01N 33/53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords:temporal, plural, detection, analyte, reagent, nucleic acid, label, conjugate, signal, optical

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2011-0294135 A1 (CARLSON) 01 December 2011 See claims 1, 10, 13 and 24; paragraphs 0067, 0109, 0115 and 0119; and figure 1.	1-3,70-73
A	BRENNER et al., `Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays`, Nature Biotechnology, Vol. 18, pp. 630-634 (31 June 2000) See figure 2.	1-3,70-73
A	US 2009-0208965 A1 (TAFAS et al.) 20 August 2009 See the whole document.	1-3,70-73
A	KR 10-2008-0003402 A (MYCOSOL, INC.) 07 January 2008 See the whole document.	1-3,70-73
A	HAN et al., `Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules', Nature Biotechnology, Vol. 19, pp. 631-635 (31 July 2001) See the whole document.	1-3,70-73

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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

Date of the actual completion of the international search	Date of mailing of the international search report
05 April 2013 (05.04.2013)	**08 April 2013 (08.04.2013)**
Name and mailing address of the ISA/KR Korean Intellectual Property Office 189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer KIM, Seung Beom Telephone No. 82-42-481-3371

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/071398

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

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

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

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

See extra sheet.

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

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

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

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

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

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

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

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(Contination of Box No.II)

2. Claims Nos.: 9, 12, 15, 23-24, 31, 36, 38, 40-41, 45, 47, 54-55, 59, 61, 75, 77, 82, 84, 92, 97, 99, 109 and 114

Claims 9, 12, 15, 23-24, 31, 36, 38, 40-41, 45, 47, 54-55, 59, 61, 75, 77, 82, 84, 92, 97, 99, 109 and 114 do not comply with PCT Article 6 because they are referring to multiple dependent claims which do not comply with PCT Rule 6.4(a).

3. Claims Nos.: 4-8, 10-11, 13-14, 16-22, 25-30, 32-35, 37, 39, 42-44, 46, 48-53, 56-58, 60, 62-69, 74, 76, 78-81, 83, 85-91, 93-96, 98, 100-108, 110-113 and 115-117

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US2012/071398

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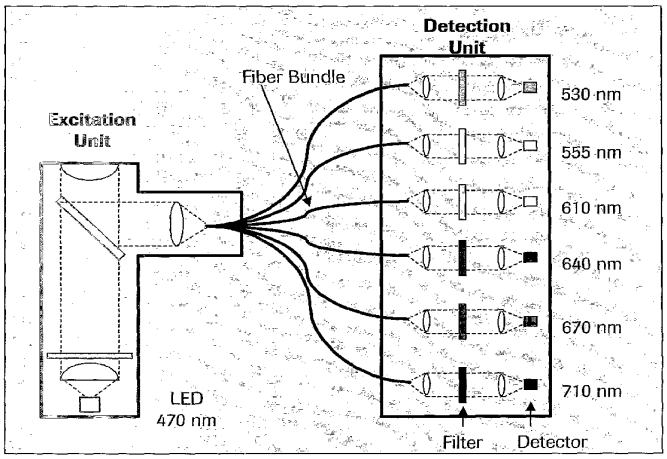
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[Continued on next page]

(54) Title: IMPROVED SYSTEM FOR MULTI COLOR REAL TIME PCR



(57) Abstract: The invention is directed to a system for performing multi-color real time PCT, comprising a flexible real time PCT instrument and a specific composition or reaction mixture for performing multiplex PCR: In particular, the present invention is directed to a composition or reaction mixtuer which comprises at least 3, preferably 4-5 and most preferably exactly 4 pairs of FRET hybridization probes. Each pair of said hybridization probes consists of a FRET donor probe carrying a FRET donor moiety and a FRET acceptor probe carrying a FRET acceptor moiety having an emission maximum between 550 and 710 nm.

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(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Improved system for multi color real time PCR

Field of invention

The present invention relates to the field of Real time PCR. In particular, the present invention is directed to a system for performing multiplex real time PCR.

Prior art background

Amplification of DNA by polymerase chain reaction (PCR) is a technique fundamental to molecular biology. Nucleic acid analysis by PCR requires sample preparation, amplification, and product analysis. Although these steps are usually performed sequentially, amplification and analysis can occur simultaneously. DNA dyes or fluorescent probes can be added to the PCR mixture before amplification and used to analyze PCR products during amplification. Sample analysis occurs concurrently with amplification in the same tube within the same instrument. This combined approach decreases sample handling, saves time, and greatly reduces the risk of product contamination for subsequent reactions, as there is no need to remove the samples from their closed containers for further analysis. The concept of combining amplification with product analysis has become known as "real time" PCR. See, for example, U.S. Patent No. 6,174,670.

Monitoring fluorescence during each cycle of PCR initially involved the use of ethidium bromide. (Higuchi R, G Dollinger, PS Walsh and R. Griffith, Simultaneous amplification and detection of specific DNA sequences, Bio/Technology 10 (1992) 413-417; Higuchi R, C Fockler G Dollinger and R Watson, Kinetic PCR analysis: real time monitoring of DNA amplification reactions, Bio/Technology 11 (1993) 1026-1030). In that system fluorescence is measured once per cycle as a relative measure of product concentration. Ethidium bromide detects double stranded DNA; if template is present fluorescence intensity increases with temperature cycling. Furthermore, the cycle number where an increase in fluorescence is first detected increases inversely proportionally to the log of the initial template concentration. Other fluorescent systems have been developed that are capable of providing additional data concerning the nucleic acid concentration and sequence.

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In kinetic real time PCR, the formation of PCR products is monitored in each cycle of the PCR. The amplification is usually measured in thermocyclers which have additional devices for measuring fluorescence signals during the amplification reaction.

Real time PCR instrumentation

Several types of real time detection thermocyclers are known in the art.

EP 0 640 828, for example discloses an apparatus for monitoring multiple nucleic acid amplification simultaneously. It is characterized in that it comprises a metal block thermal cycler, including a heat conducting member having multiple recesses formed therein in order to take up a multiwell plate such as a microtiter plate. Detection is obtained by means of a CCD camera, arranged for detecting light emitted (simultaneously) from all of said recesses. Alternatively, the use of fiber optics is suggested. Depending on the fluorescent dyes which are used and – more important-, depending on the presence of a filter wheel close to the CCD camera, the system has the capability of analyzing multiplex amplification reactions, wherein in one reaction chamber, one or more different amplicons are detected by 2 or more differently labeled hybridization probes. Yet, EP 0 640 828 does neither anticipate nor suggest, which labels or detection formates could be used for such a multiplex/multicolor approach.

US 6,015,674 discloses an apparatus and a system for real time PCR detection and quantification, characterized in that it is capable of detecting first and second fluorescent indicators, which may be used as labels for different hybridization probes in order to detect different amplicons in the same reaction vessel. Yet, US 6,015,674 does not disclose a system for performing multipex experiments with a higher degree of complexity.

Another typical example is the Roche Diagnostics LightCycler (Cat. No. 2 0110468). It is a fast PCR system enabling kinetic on-line PCR quantification and subsequent analysis of PCR-product melting curves. The optical system of the current LightCycler version 1.2 being commercially available contains one light source, a blue light emitting diode (470 nm LED) and three detection channels. The amplification products are detected by means of fluorescent labeled hybridization