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Application Number: 16864744

Document Date: 05/01/2020

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UTILITY PATENT APPLICATION TRANSMITTAL <i>(Only for new nonprovisional applications under 37 CFR 1.53(b))</i>	Attorney Docket No.	38075-716.304
	First Named Inventor	Emil P. Kartalov
	Title	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
	Priority Mail Express® Label No.	e-Filed on May 1, 2020

APPLICATION ELEMENTS <i>See MPEP chapter 600 concerning utility patent application contents.</i>	ADDRESS TO: Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450
1. ☐ Fee Transmittal Form (PTO/SB/17 or equivalent)	10. ☐ Assignment Papers (cover sheet & document(s)) Name of Assignee _____
2. ☐ Applicant asserts small entity status. See 37 CFR 1.27	11. ☐ 37 CFR 3.73(c) Statement (when there is an assignee) ☐ Power of Attorney
3. ☐ Applicant certifies micro entity status. See 37 CFR 1.29. Applicant must attach form PTO/SB/15A or B or equivalent.	12. ☐ English Translation Document (if applicable)
4. ☒ Specification [Total Pages <u>73</u>] Both the claims and abstract must start on a new page. (See MPEP § 608.01(a) for information on the preferred arrangement)	13. ☐ Information Disclosure Statement (PTO/SB/08 or PTO-1449) ☐ Copies of citations attached
5. ☒ Drawing(s) (35 U.S.C. 113) [Total Sheets <u>8</u>]	14. ☐ Preliminary Amendment
6. ☒ Inventor's Oath or Declaration [Total Pages <u>3</u>] (including substitute statements under 37 CFR 1.64 and assignments serving as an oath or declaration under 37 CFR 1.63(e))	15. ☐ Return Receipt Postcard (MPEP § 503) (Should be specifically itemized)
a. ☐ Newly executed (original or copy)	16. ☐ Certified Copy of Priority Document(s) (if foreign priority is claimed)
b. ☒ A copy from a prior application (37 CFR 1.63(d))	17. ☐ Nonpublication Request Under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent.
7. ☒ Application Data Sheet * See note below. See 37 CFR 1.76 (PTO/AIA/14 or equivalent)	18. ☒ Other: Request for Transfer of a Computer Readable form
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	

*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: <u>021971</u> OR ☐ Correspondence address below					
Name					
Address					
City	State	Zip Code			
Country	Telephone	Email			
Signature	/Nicholas Tiee/			Date	May 1, 2020
Name (Print/Type)	Nicholas Tiee			Registration No. (Attorney/Agent)	77,907

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

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

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	38075-716.304
		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					

Application Data Sheet 37 CFR 1.76		Attorney Docket Number		38075-716.304	
		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	38075-716.304	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.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	38075-716.304
		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	15/914356	2018-03-07		
Prior Application Status	Patented		Remove		
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
15/914356	Continuation of	14/451876	2014-08-05	10068051	2018-09-04
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.					Add

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	38075-716.304
		Application Number	
Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS		

Foreign Priority Information:

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

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

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.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	38075-716.304
		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.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	38075-716.304
		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	Remove	
If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.			
		Clear	
☐ Assignee	Legal Representative under 35 U.S.C. 117		Joint Inventor
Person to whom the inventor is obligated to assign.		Person who shows sufficient proprietary interest	
If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:			
Name of the Deceased or Legally Incapacitated Inventor:			
If the Applicant is an Organization check here. ☒			
Organization Name	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. Add			

Assignee Information including Non-Applicant Assignee Information:

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

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	38075-716.304
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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.				
				Remove
If the Assignee or Non-Applicant Assignee is an Organization check here.				☐
Prefix	Given Name	Middle Name	Family Name	Suffix
Mailing Address Information For Assignee including Non-Applicant Assignee:				
Address 1				
Address 2				
City		State/Province		
Country i		Postal Code		
Phone Number		Fax Number		
Email Address				
Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.				Add

Signature:

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	/Nicholas Tiee/		Date (YYYY-MM-DD)	2020-05-01
First Name	Nicholas	Last Name	Tiee	Registration Number
				77907
Additional Signature may be generated within this form by selecting the Add button.				Add

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	38075-716.304
		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 Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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
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Assignee: California Institute of Technology
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(650) 493-9300
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Electronically Filed on May 1, 2020

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

CROSS-REFERENCE

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

SEQUENCE LISTING

[0001.1] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on August 20, 2014, is named 38075-716-301-Seqlist.txt and is 9,260 bytes in size.

STATEMENT AS TO FEDERALLY SPONSERED RESEARCH

[0001.5] This invention was made with the support of the United States government under Grant No. 1144469 awarded by the National Science Foundation. The government has certain rights in the invention.

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.

[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.

[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 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.

[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 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 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 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 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.

[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 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 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. 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. 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 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 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).

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				Analyte(s) Present
Blue	Green	Yellow	Red	
1	0	0	0	p
2	0	0	1	pA
2	0	1	0	pB
2	1	0	0	pC
2	0	1	1	pD
2	1	0	1	pE
2	1	1	0	pF
2	1	1	1	pG
3	0	1	1	pAB
3	1	0	1	pAC
3	0	1	2	pAD
3	1	0	2	pAE
3	1	1	1	pAF, pCD, pBE
3	1	1	2	pAG, pDE
3	1	1	0	pBC
3	0	2	1	pBD
3	1	2	0	pBF
3	1	2	1	pBG, pDF
3	2	0	1	pCE
3	2	1	0	pCF
3	2	1	1	pCG, pEF
3	1	2	2	pDG
3	2	1	2	pEG
3	2	2	1	pFG
4	1	1	1	pACB
4	0	2	2	pABD
4	2	2	0	pBCF
4	2	0	2	pACE
4	1	1	2	pABE, pACD
4	1	2	1	pABF, pBCD
4	2	1	1	pACF, pBCE
4	1	2	2	pADF, pABG, pBDE
4	2	1	2	pACG, pCDE, pAEF
4	2	2	1	pBCG, pBEF, pCDF
4	2	2	2	pCDG, pAFG, pBEG, pDEF

4	1	1	3	pADE
4	1	3	1	pBDF
4	3	1	1	pCEF
4	1	2	3	pADG
4	3	2	1	pCFG
4	2	1	3	pAEG
4	2	3	1	pBFG
4	1	3	2	pBDG
4	3	1	2	pCEG
4	2	2	3	pDEG
4	3	2	2	pEFG
5	1	2	2	pABCD
5	2	1	2	pABCE
5	2	2	1	pABCF
5	2	2	2	pABCG, pABEF, pBCDE, pACDF
5	1	2	3	pABDE
5	2	1	3	pACDE
5	1	3	2	pABDF
5	2	1	3	pBCDF
5	3	1	2	pACEF
5	3	2	1	pBCEF
5	2	2	3	pABEG, pACDG, pADEF
5	2	3	2	pABFG, pBCDG, pBDEF
5	3	2	2	pACFG, pBCEG, pCDEF
5	1	3	3	pABDG
5	3	1	3	pACEG
5	3	3	1	pBCFG
5	2	3	3	pADFG, pBDEG
5	3	3	2	pBEFG, pDEFG
5	3	2	3	pCDEG, pAEFG
5	2	2	4	pADEG
5	2	4	2	pBDFG
5	4	2	2	pCEFG
5	3	3	3	pDEFG
6	2	2	3	pABCDE
6	2	3	2	pABCDF
6	3	2	2	pABCEF
6	2	3	3	pABCDG, pABDEF
6	3	2	3	pABCEG, pACDEF
6	3	3	2	pABCFG, pBCDEF
6	4	3	2	pBCEFG
6	4	2	3	pACEFG
6	3	3	3	pABEFG, pACDFG, pBCDEG
6	3	4	2	pBCDFG
6	2	4	3	pABDFG
6	3	2	4	pACDEG

6	2	3	4	pABDEG
6	4	3	3	pCDEFG
6	3	4	3	pBDEFG
6	3	3	4	pADEFG
7	4	4	3	pBCDEFG
7	4	3	4	pACDEFG
7	3	4	4	pABDEFG
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

[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

[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 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
<i>Plasmodium</i>	1	0	1	0
HIV	1	1	0	0
<i>Mycobacterium</i>	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 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 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 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	
	~~Ⓢ~~	~~1~~	~~0~~	~~1~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	E	1	1	0	1	
	~~Ⓢ~~	~~1~~	~~1~~	~~0~~	~~1~~	Eliminated from coding scheme to eliminate degeneracy.
	G	1	1	1	1	
<i>Cumulative Maximum Result</i>		<i>6</i>	<i>3</i>	<i>2</i>	<i>3</i>	
2	H	1	0	0	4	
	I	1	0	3	0	
	J	1	4	0	0	
	~~Ⓢ~~	~~1~~	~~4~~	~~0~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	L	1	4	0	4	
	~~Ⓢ~~	~~1~~	~~4~~	~~0~~	~~4~~	Eliminated from coding scheme to eliminate degeneracy.
	N	1	4	3	4	
<i>Cumulative Maximum Result</i>		<i>11</i>	<i>15</i>	<i>8</i>	<i>15</i>	
3	O	1	0	0	16	
	P	1	0	9	0	
	Q	1	16	0	0	
	~~Ⓢ~~	~~1~~	~~0~~	~~9~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	S	1	16	0	16	
	~~Ⓢ~~	~~1~~	~~16~~	~~0~~	~~16~~	Eliminated from coding scheme to eliminate degeneracy.
	U	1	16	9	16	
<i>Cumulative Maximum Result</i>						

Cumulative Maximum Result for Three Non- Degenerate Tiers		16	63	26	63	
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[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 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~~	~~2~~	~~3~~	~~4~~	Eliminated from coding scheme to eliminate degeneracy.
<i>Cumulative Maximum Result</i>		6	3	2	3	
2	Seq 7	1	4	0	0	
	Seq 8	1	0	3	0	
	Seq 9	1	0	0	4	
<i>Etc. - i.e. Continue as provided in Table 6.</i>						

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, 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
		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 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. 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 (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 genera *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 parvotuberculosis*, *Bordetella pertussis*, *Borrelia burgdorferi*, *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella suis*, *Campylobacter jejuni*, *Chlamydia pneumoniae*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *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 genera *Acanthamoeba*, *Babesia*, *Balamuthia*, *Balantidium*, *Blasocystis*, *Cryptosporidium*, *Dientamoeba*, *Entamoeba*, *Giardia*, *Isospora*, *Leishmania*, *Naegleria*, *Pediculus*, *Plasmodium*, *Rhinosporidium*, *Sarcocystis*, *Schistosoma*, *Toxoplasma*, *Trichomonas*, and *Trypanosoma*. Specific examples of parasites include *Babesia divergens*, *Babesia bigemina*, *Babesia equi*, *Babesia microfti*, *Babesia duncani*, *Balamuthia mandrillaris*, *Balantidium coli*, *Dientamoeba fragilis*, *Entamoeba histolytica*, *Giardia lamblia*, *Isospora belli*, *Naegleria fowleri*, *Pediculus humanus*, *Plasmodium falciparum*, *Plasmodium knowlesi*, *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium vivax*, *Rhinosporidium seeberi*, *Sarcocystis bovi hominis*, *Sarcocystis sui hominis*, *Schistosoma mansoni*, *Toxoplasma gondii*, *Trichomonas vaginalis*, *Trypanosoma brucei*, and *Trypanosoma cruzi*.

[00113] Examples of fungi include those from the genera *Apophysomyces*, *Aspergillus*, *Blastomyces*, *Candida*, *Cladosporium*, *Coccidioides*, *Cryptococcus*, *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*, *Cryptococcus 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.

[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, 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.

[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 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 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.

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

[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, 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 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 use 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, 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.

[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, thiocarbocyanine, 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, phthalocyanine, 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 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. 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.

[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.

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.

[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

[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 scheme 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.

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 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 (SEQ ID NO: 1)

Forward Primer (3' to 5')	GGAAGCTCTATTAGATACAGGAGCAG <u>(SEQ ID NO: 2)</u>
Reverse Primer (3' to 5')	CCAATTATGTTGACAGGTGTAGGTCC <u>(SEQ ID NO: 3)</u>
Probe 1 (3' to 5')	/56-FAM/TGAGTTTGCCAGGAAGATGGAAACCA/3BHQ_1/ <u>(SEQ ID NO: 4)</u>

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')	CAGCTACAACCATCCCTTCAGACAGGATCAGAAGAAGCTTAGATCATTATA TAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAG ACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAG AAAAAAGCACAGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGTCA <u>(SEQ ID NO: 5)</u>
Forward Primer (3' to 5')	CAGCTACAACCATCCCTTCAGACA <u>(SEQ ID NO: 6)</u>
Reverse Primer (3' to 5')	TGACCTGATTGCTGTGTCCTGTGT <u>(SEQ ID NO: 7)</u>
Probe 1 (3' to 5')	/56-FAM/AGCAACCCTCTATTGTGTGCATCAAAGG/3BHQ_1 <u>(SEQ ID NO: 8)</u>
Probe 2 (3' to 5')	/5Cy3/AAAGCACAGCAAGCAGCAGCTGA/3BHQ_2/ <u>(SEQ ID NO: 9)</u>

Table 11. *Plasmodium* ChR7 sequence information.

Sequence Name	<i>Plasmodium</i> ChR7 199mer synthesized from bases 1139138 - 1141223
Source	UCSC <i>Plasmodium falciparum</i> Genome Browser Gateway
Target sequence (3' to 5')	GCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTTCGATTCCGGAG AGGGAGCCTGAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTA AATTACCCAATTCTAAAGAAGAGAGGTAGTGACAAGAAATAACAATGCAA GGCCAATTTAAACCTTCCAGAGTAACAATTGGAGGGCAAGTCTGGTG <u>(SEQ ID NO: 10)</u>
Forward Primer (3' to 5')	GCCTAACATGGCTATGACGGGTAA <u>(SEQ ID NO: 11)</u>
Reverse Primer (3' to 5')	CACCAGACTTGCCCTCCAATTGTT <u>(SEQ ID NO: 12)</u>

Probe 1 (3' to 5')	/56-FAM/ATTCCGGAGAGGGAGCCTGAGAAATA/3BHQ_1/ (SEQ ID NO: 13)
Probe 2 (3' to 5')	/56-ROXN/AAGGAAGGCAGCAGGCGCGTAAATTA/3BHQ_2/ (SEQ ID NO: 14)

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')	TCAGCCCATCCTCCTTCGGCAGTATGGAGGGTGTGCGGGCGGCGAGCCGC CGTCCCCAAAGACGTGCGGGTCGTACACGTACACGTACCAGGGCGGCGGG CCTCCGACCCGGTACGCTCTCGTAAATGCTTCCCTGCTGGTGCCGATCTG GGACCGCGCCGCGGAGACATTTCAGTACCAGATCGAACTCGG (SEQ ID NO: 15)
Forward Primer (3' to 5')	TCAGCCCATCCTCCTTCGGCAGTAT (SEQ ID NO: 16)
Reverse Primer (3' to 5')	CCGAGTTCGATCTGGTACTCGAATGT (SEQ ID NO: 17)
Probe 1 (3' to 5')	/56-FAM/AAAGACGTGCGGGTCGTACACGTACA/3BHQ_1/ (SEQ ID NO: 18)
Probe 2 (3' to 5')	/5Cy5/TAAATGCTTCCCTGCTGGTGCCGAT/3IAbRQSp/ (SEQ ID NO: 19)

Table 13. *Mycobacterium tuberculosis* rpoB sequence information

Sequence Name	Mycobacterium Tuberculosis rpoB 200mer synthesized from Mycobacterium tuberculosis rpoB genome
Source	EMBL Bank GQ395623
Target sequence (3' to 5')	GAGTGCAAAGACAAGGACATGACGTACGCGGCCCCGCTGTTTCGTACGGC CGAGTTCATCAACAACAACACCGGCGAGATCAAGAGCCAGACGGTGTTC TGGGTGACTTCCCGATGATGACCGAGAAGGGCACCTTCATCATCAACGGC ACCGAGCGCGTCGTGGTCAGCCAGCTGGTCCGCTCGCCCGGTGTGTACTT (SEQ ID NO: 20)
Forward Primer (3' to 5')	GAGTGCAAAGACAAGGACATGACG (SEQ ID NO: 21)
Reverse Primer (3' to 5')	AAGTACACACCGGGCGAGC (SEQ ID NO: 22)
Probe 1 (3' to 5')	/56-FAM/CGCTGTTTCGTACGGCCGAGTTCAT/3BHQ_1/ (SEQ ID NO: 23)
Probe 2 (3' to 5')	/5Cy3/AGATCAAGAGCCAGACGGTGTTCATG/3BHQ_2/ (SEQ ID NO: 24)
Probe 3 (3' to 5')	/5Cy5/AAGGGCACCTTCATCATCAACGGCA/3IAbRQSp/ (SEQ ID NO: 25)

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 AATGCTTGTGAGAAATCCACTCTCACGAACTCCACGCACGAAATGTATT GGATATCCAATGGTACAGGCAACATCGTCTCATTACAATGACACACAGG AGACCCACCATAGAGAAAGATGTGGATTTAGGAGCAGGAACCCGACATGT <u>(SEQ ID NO: 26)</u>
Forward Primer (3' to 5')	ATGCCAACTGTGATTGAGCACT <u>(SEQ ID NO: 27)</u>
Reverse Primer (3' to 5')	ACATGTCGGGTTCTGCTCCTAAA <u>(SEQ ID NO: 28)</u>
Probe 1 (3' to 5')	/56-FAM/ACAAAGGAAACATGGAGGAATGCTTGTGA/3BHQ_1/ <u>(SEQ ID NO: 29)</u>
Probe 2 (3' to 5')	/5Cy3/ACTCTCACGAACTCCACGCACGAAA/3BHQ_2/ <u>(SEQ ID NO: 30)</u>
Probe 3 (3' to 5')	/56-ROXN/ACAATGACACACAGGAGACCCACCAT/3BHQ_2/ <u>(SEQ ID NO: 31)</u>
Probe 4 (3' to 5')	/5Cy5/GGATATCCAATGGTACAGGCAACATCGT/3IAbRQSp/ <u>(SEQ ID NO: 32)</u>

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). 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 μ L
		Taq 5x Master Mix	-	42 μ L
	Template	Poly Protease Template	17 nM	2 μ L
	Primers	Poly Protease FWD Primer	1 nM	2 μ L
		Protease RVS Primer	1 nM	2 μ L
	Probe	Poly Protease Probe 1	1 nM	2 μ L
2		UltraPure Water	-	28 μ L
		Taq 5x Master Mix	-	42 μ L
	Templates	Malaria Template	15 nM	2 μ L
		Herpes Template	17 nM	2 μ L
		TB Template	16 nM	2 μ L
		Dengue Virus Template	17 nM	2 μ L
	Primers	Poly Protease FWD Primer	1 nM	2 μ L
		Protease RVS Primer	1 nM	2 μ L
	Probe	Poly Protease Probe 1	1 nM	2 μ L
3		UltraPure Water	-	32 μ L
		Taq 5x Master Mix	-	42 μ L
	Template	Malaria Template	15 nM	2 μ L
	Primers	Malaria FWD Primer	1 μ M	2 μ L
		Malaria RVS Primer	1 μ M	2 μ L
	Probes	Malaria Probe 1	1 μ M	2 μ L
		Malaria Probe 2	1 μ M	2 μ L
4		UltraPure Water	-	26 μ L
		Taq 5x Master Mix	-	42 μ L
	Templates	Poly Protease Template	17 nM	2 μ L
		Herpes Template	17 nM	2 μ L
		TB Template	16 nM	2 μ L
		Dengue Virus Template	17 nM	2 μ L
	Primers	Malaria FWD Primer	1 μ M	2 μ L
		Malaria RVS Primer	1 μ M	2 μ L
	Probes	Malaria Probe 1	1 μ M	2 μ L
		Malaria Probe 2	1 μ M	2 μ L
5		UltraPure Water	-	32 μ L
		Taq 5x Master Mix	-	42 μ L
	Template	Herpes Template	17 nM	2 μ L
	Primers	Herpes FWD Primer	1 μ M	2 μ L
		Herpes RVS Primer	1 μ M	2 μ L

	<i>Probes</i>	Herpes Probe 1	1 μ M	2 μ L
		Herpes Probe 2	1 μ M	2 μ L
6		UltraPure Water	-	26 μ L
		Taq 5x Master Mix	-	42 μ L
	<i>Templates</i>	Poly Protease Template	17 nM	2 μ L
		Malaria Template	15 nM	2 μ L
		TB Template	16 nM	2 μ L
		Dengue Virus Template	17 nM	2 μ L
	<i>Primers</i>	Herpes FWD Primer	1 μ M	2 μ L
		Herpes RVS Primer	1 μ M	2 μ L
	<i>Probes</i>	Herpes Probe 1	1 μ M	2 μ L
		Herpes Probe 2	1 μ M	2 μ L
7		UltraPure Water	-	30 μ L
		Taq 5x Master Mix	-	42 μ L
	<i>Template</i>	TB Template	16 nM	2 μ L
	<i>Primers</i>	TB FWD Primer	1 μ M	2 μ L
		TB RVS Primer	1 μ M	2 μ L
	<i>Probes</i>	TB Probe 1	1 μ M	2 μ L
		TB Probe 2	1 μ M	2 μ L
		TB Probe 3	1 μ M	2 μ L
8		UltraPure Water	-	30 μ L
		Taq 5x Master Mix	-	42 μ L
	<i>Templates</i>	Poly Protease Template	17 nM	2 μ L
		Malaria Template	15 nM	2 μ L
		Herpes Template	17 nM	2 μ L
		Dengue Virus Template	17 nM	2 μ L
	<i>Primers</i>	TB FWD Primer	1 μ M	2 μ L
		TB RVS Primer	1 μ M	2 μ L
	<i>Probes</i>	TB Probe 1	1 μ M	2 μ L
		TB Probe 2	1 μ M	2 μ L
		TB Probe 3	1 μ M	2 μ L
9		UltraPure Water	-	28 μ L
		Taq 5x Master Mix	-	42 μ L
	<i>Template</i>	Dengue Virus Template	17 nM	2 μ L
	<i>Primers</i>	Dengue Virus FWD Primer	1 μ M	2 μ L
		Dengue Virus RVS Primer	1 μ M	2 μ L
	<i>Probes</i>	Dengue Virus Probe 1	1 μ M	2 μ L
		Dengue Virus Probe 2	1 μ M	2 μ L
		Dengue Virus Probe 3	1 μ M	2 μ L
		Dengue Virus Probe 4	1 μ M	2 μ L
10		UltraPure Water	-	22 μ L
		Taq 5x Master Mix	-	42 μ L
	<i>Templates</i>	Poly Protease Template	17 nM	2 μ L
		Malaria Template	15 nM	2 μ L
		Herpes Template	17 nM	2 μ L
		TB Template	16 nM	2 μ L

	<i>Primers</i>	Dengue Virus FWD Primer	1 μ M	2 μ L
		Dengue Virus RVS Primer	1 μ M	2 μ L
	<i>Probes</i>	Dengue Virus Probe 1	1 μ M	2 μ L
		Dengue Virus Probe 2	1 μ M	2 μ L
		Dengue Virus Probe 3	1 μ M	2 μ L
		Dengue Virus Probe 4	1 μ M	2 μ L
		Taq 5x Master Mix	-	42 μ L
11	<i>Templates</i>	Poly Protease Template	17 nM	2 μ L
		Malaria Template	15 nM	2 μ L
		Herpes Template	17 nM	2 μ L
		Dengue Virus Template	17 nM	2 μ L
	<i>Primers</i>	Poly Protease FWD Primer	1 μ M	2 μ L
		Poly Protease RVS Primer	1 μ M	2 μ L
		Malaria FWD Primer	1 μ M	2 μ L
		Malaria RVS Primer	1 μ M	2 μ L
		Herpes FWD Primer	1 μ M	2 μ L
		Herpes RVS Primer	1 μ M	2 μ L
		Dengue Virus FWD Primer	1 μ M	2 μ L
		Dengue Virus RVS Primer	1 μ M	2 μ L
	<i>Probes</i>	Poly Protease Probe 1	1 μ M	2 μ L
		Malaria Probe 1	1 μ M	2 μ L
		Malaria Probe 2	1 μ M	2 μ L
		Herpes Probe 1	1 μ M	2 μ L
		Herpes Probe 2	1 μ M	2 μ L
		Dengue Virus Probe 1	1 μ M	2 μ L
		Dengue Virus Probe 2	1 μ M	2 μ L
		Dengue Virus Probe 3	1 μ M	2 μ L
		Dengue Virus Probe 4	1 μ M	2 μ L
12		UltraPure Water	-	2 μ L
		Taq 5x Master Mix	-	42 μ L
	<i>Templates</i>	Poly Protease Template	17 nM	2 μ L
		Malaria Template	15 nM	2 μ L
		Herpes Template	17 nM	2 μ L
		TB Template	16 nM	2 μ L
	<i>Primers</i>	Poly Protease FWD Primer	1 μ M	2 μ L
		Poly Protease FWD Primer	1 μ M	2 μ L
		Malaria FWD Primer	1 μ M	2 μ L
		Malaria RVS Primer	1 μ M	2 μ L
		Herpes FWD Primer	1 μ M	2 μ L
		Herpes RVS Primer	1 μ M	2 μ L
		TB FWD Primer	1 μ M	2 μ L
		TB RVS Primer	1 μ M	2 μ L
	<i>Probes</i>	Poly Protease Probe 1	1 μ M	2 μ L
		Malaria Probe 1	1 μ M	2 μ L
		Malaria Probe 2	1 μ M	2 μ L
		Herpes Probe 1	1 μ M	2 μ L

		Herpes Probe 2	1 μ M	2 μ L
		TB Probe 1	1 μ M	2 μ L
		TB Probe 2	1 μ M	2 μ L
		TB Probe 3	1 μ M	2 μ L
13		UltraPure Water	-	12 μ L
		Taq 5x Master Mix	-	42 μ L
	<i>Templates</i>	Poly Protease Template	17 nM	2 μ L
		Herpes Template	17 nM	2 μ L
		TB Template	16 nM	2 μ L
	<i>Primers</i>	Poly Protease FWD Primer	1 μ M	2 μ L
		Poly Protease FWD Primer	1 μ M	2 μ L
		Herpes FWD Primer	1 μ M	2 μ L
		Herpes RVS Primer	1 μ M	2 μ L
		TB FWD Primer	1 μ M	2 μ L
		TB RVS Primer	1 μ M	2 μ L
	<i>Probes</i>	Poly Protease Probe 1	1 μ M	2 μ L
		Herpes Probe 1	1 μ M	2 μ L
		Herpes Probe 2	1 μ M	2 μ L
		TB Probe 1	1 μ M	2 μ L
		TB Probe 2	1 μ M	2 μ L
		TB Probe 3	1 μ M	2 μ L
14		UltraPure Water	-	10 μ L
		Taq 5x Master Mix	-	42 μ L
	<i>Templates</i>	Poly Protease Template	17 nM	2 μ L
		Malaria Template	15 nM	2 μ L
		Dengue Virus Template	17 nM	2 μ L
	<i>Primers</i>	Poly Protease FWD Primer	1 μ M	2 μ L
		Poly Protease FWD Primer	1 μ M	2 μ L
		Malaria FWD Primer	1 μ M	2 μ L
		Malaria RVS Primer	1 μ M	2 μ L
		Dengue Virus FWD Primer	1 μ M	2 μ L
		Dengue Virus RVS Primer	1 μ M	2 μ L
	<i>Probes</i>	Poly Protease Probe 1	1 μ M	2 μ L
		Malaria Probe 1	1 μ M	2 μ L
		Malaria Probe 2	1 μ M	2 μ L
		Dengue Virus Probe 1	1 μ M	2 μ L
		Dengue Virus Probe 2	1 μ M	2 μ L
		Dengue Virus Probe 3	1 μ M	2 μ L
		Dengue Virus Probe 4	1 μ M	2 μ L

[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
<i>Plasmodium</i>	1	0	1	0
HSV	1	0	0	1
<i>Mycobacterium</i>	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 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.

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
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
Dengue Virus Probe 1	200nM	4µL

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

Reagents	Concentration	Volume Added
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL

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

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

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

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

Table 22. Binary FAM Experiment 5 Cocktail: dengue virus

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

Table 23. Binary FAM Experiment 6 Cocktail: HIV p17

Reagents	Concentration	Volume Added
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	-

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

Table 24. Binary FAM Experiment 7 Cocktail: HIV polyprotease

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

P17 Probe 1	400nM	4μL
Dengue Virus Probe 1	200nM	4μL

[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 polypotease (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 polypotease. A result of 5 indicates the presence of dengue virus and HIV polypotease, and the absence of HIV p17. Finally a result of 6 indicates the presence of HIV p17 and HIV polypotease, and the absence of dengue virus. This coding scheme can be extended to encode additional analytes, as described elsewhere in this disclosure.

CLAIMS

WHAT IS CLAIMED IS:

1. A kit for use in detecting the presence or absence of each of M analytes of a plurality of analytes in a single volume, in any combination of presence or absence, the kit comprising x analyte-specific reagents, packaging, and instructions, each of the F analyte-specific reagents 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 M and F are integers and M has a value that is greater than that of F.
2. The kit of claim 1, wherein said analyte-specific reagents correspond with said analytes, and wherein said analytes are encoded a first value of a signal, thus generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, further wherein said encoding is performed in a manner that reduces or eliminates degeneracy.
3. The kit of claim 1, wherein use of said kit detects 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.
4. The kit of claim 3, wherein said first value is an intensity or range of intensities.
5. The kit of claim 4, wherein said second value is a wavelength or a range of wavelengths.
6. The kit of claim 1, wherein use of said kit detects the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, without immobilization, separation, or melting curve analysis of said analytes.
7. A system for detecting the presence or absence of M analytes in a plurality of analytes, comprising:
 - a. a first computer-readable software component configured for encoding each of said M analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said M analytes is represented in said coding scheme by said first value;
 - b. F analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said M analytes is present in said sample;
 - c. an instrument configured for measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and

- d. a second computer-readable software component configured for determining whether each of said M analytes is present or absent based on said cumulative measurement and said coding scheme,

wherein F and M are integers and M is greater than F.

- 8. The system of claim 7, wherein said system 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.
- 9. The system of claim 7, wherein said first value is an intensity or range of intensities,
 - e. wherein said first computer-readable software is further configured for encoding each of said analytes as a second value of said signal, thereby generating said coding scheme;
 - f. said instrument is further configured for measuring said second values of said signal within said sample, thereby providing said cumulative measurement; and
 - g. said second computer-readable software is further configured for determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.
- 10. The system of claim 9, wherein said first value is an intensity or a range of intensities.
- 11. The system of claim 10, wherein said second value is a wavelength or a range of wavelengths.
- 12. The system of claim 7, wherein said system is capable of detecting the presence or absence of each of at least six analytes of said analytes in a single volume, in any combination of presence or absence, without immobilization, separation, or melting curve analysis of said analytes.
- 13. The system of claim 9, wherein said system 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.
- 14. 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 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 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.
15. The method of claim 14, wherein said method detects 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.
16. The method of claim 14, wherein said first value is an intensity or range of intensities.
17. The method of claim 15, wherein said second value is a wavelength or a range of wavelengths.
18. The method of claim 15, wherein use of said kit detects the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, without immobilization, separation, or melting curve analysis of said analytes.
19. 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 reduces or eliminates degeneracy;
 - b. providing a sample comprising, or potentially comprising, at least one of said analytes;
 - c. contacting a sample that has been provided comprising at least one of said analytes(s) 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,

wherein said first value is an intensity or range of intensities and said first value is incremented in said coding scheme to an amount greater than the sum of said preceding first values.

20. The method of claim 19, wherein said first value is an intensity or range of intensities, wherein said second value is a wavelength or a range of wavelengths, and wherein said method detects the presence or absence of each of at least six of said analytes in a single volume, in any combination of presence or absence, without immobilization, separation, or melting curve analysis of said analytes.

21. An assay that is capable of unambiguously detecting the presence or absence of each of at least M analytes,

wherein each of said M analytes is encoded as a value of one component of a fluorescent signal,

wherein said fluorescent signal comprises F wavelength components, and

wherein said assay is capable of detecting the presence or absence of each of said at least M analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis, based on detection of said F wavelength components, when M is greater than F.

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.

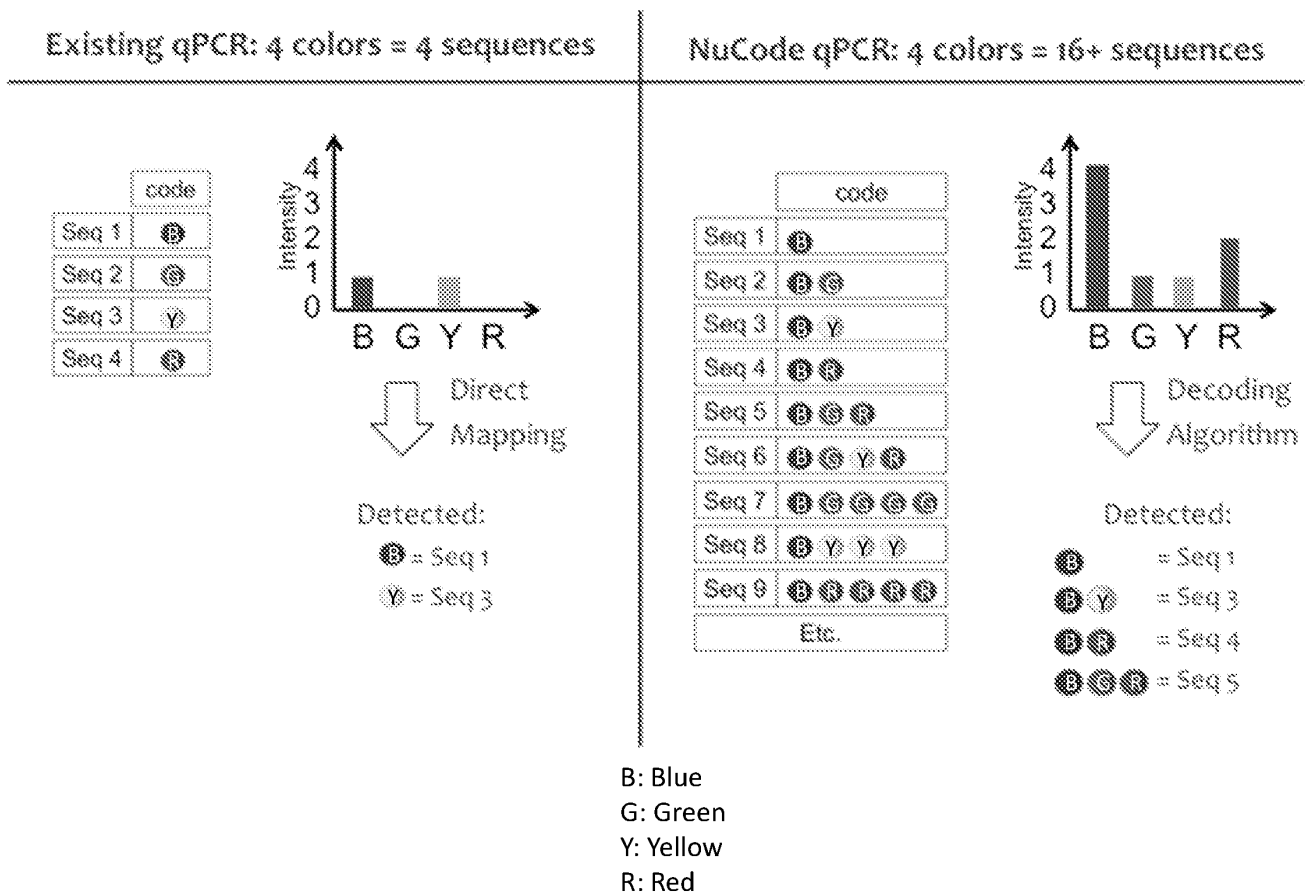
Fig. 1

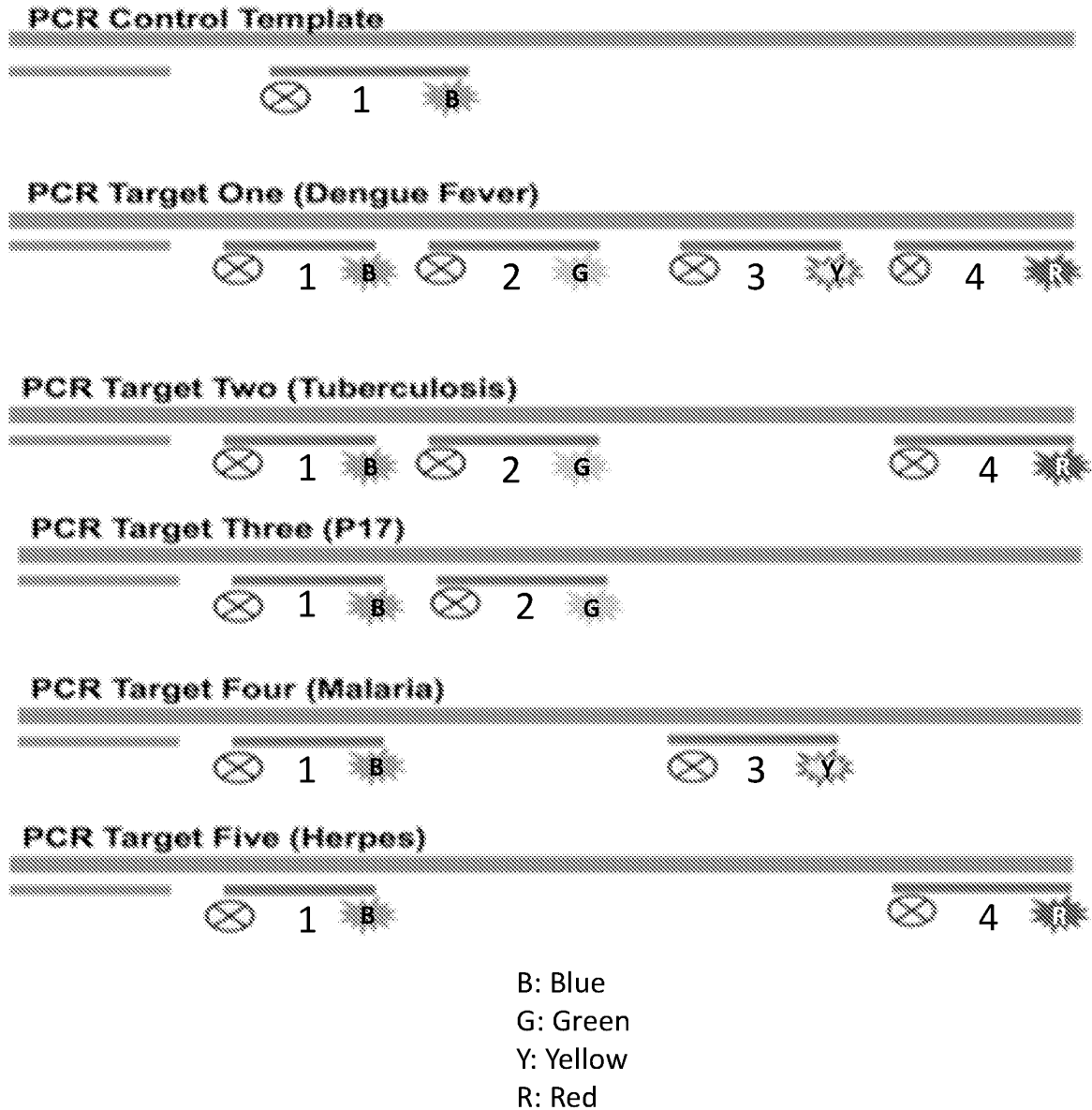
Fig. 2

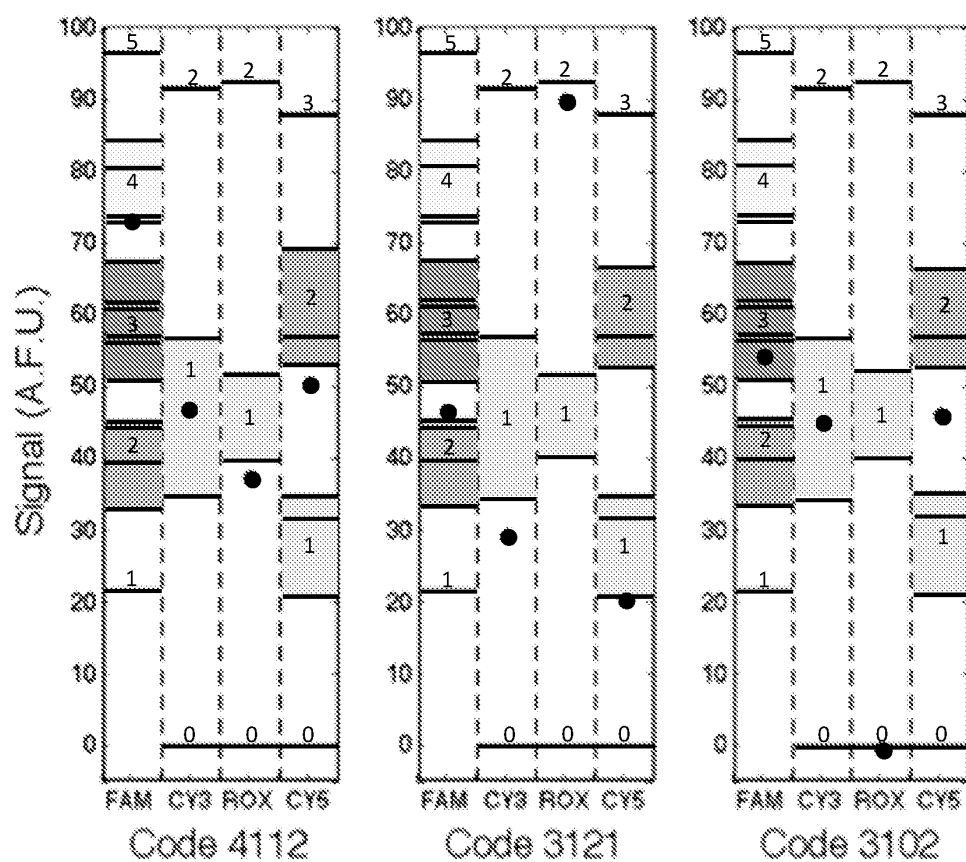
Fig. 3

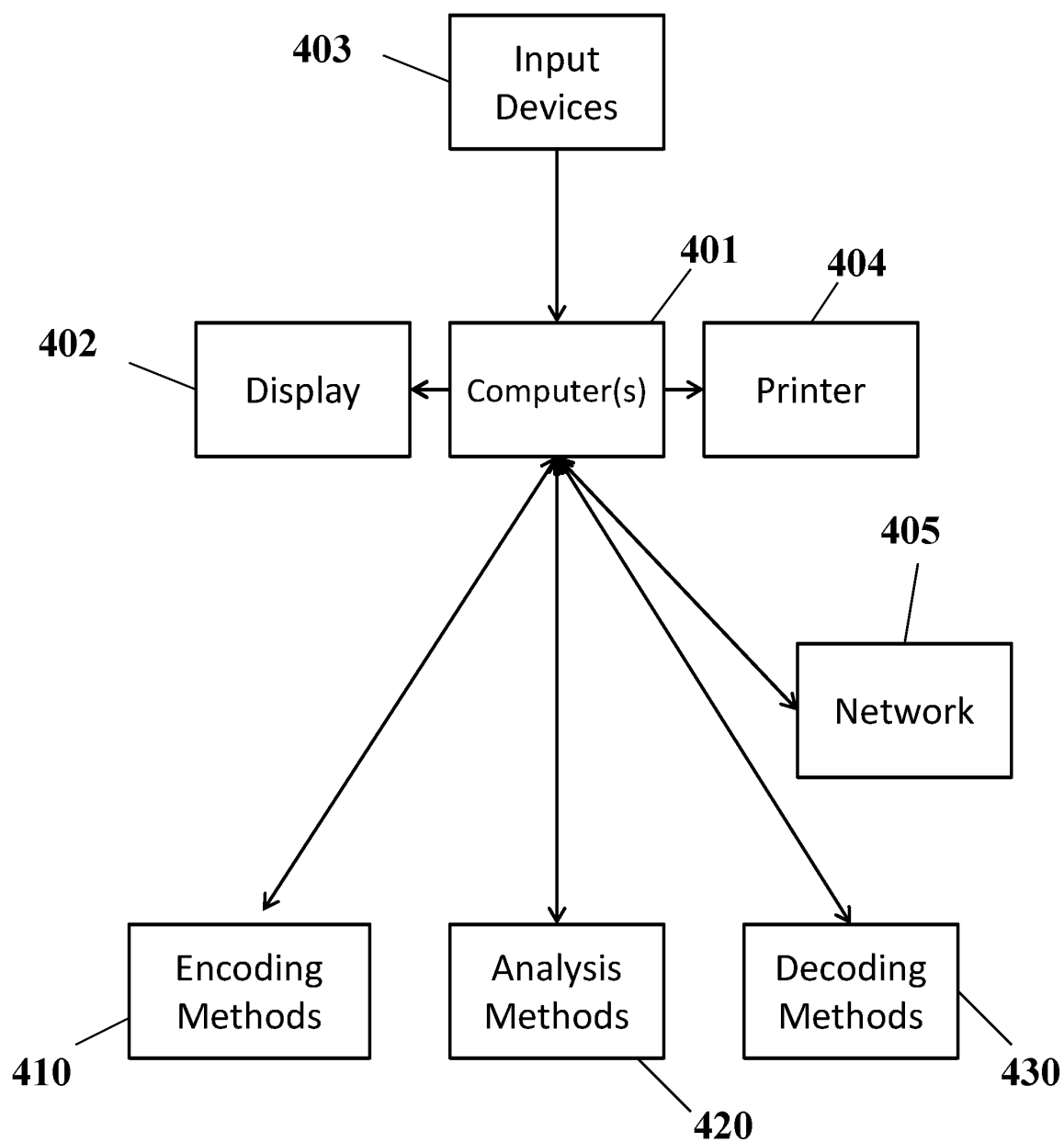
Fig. 4

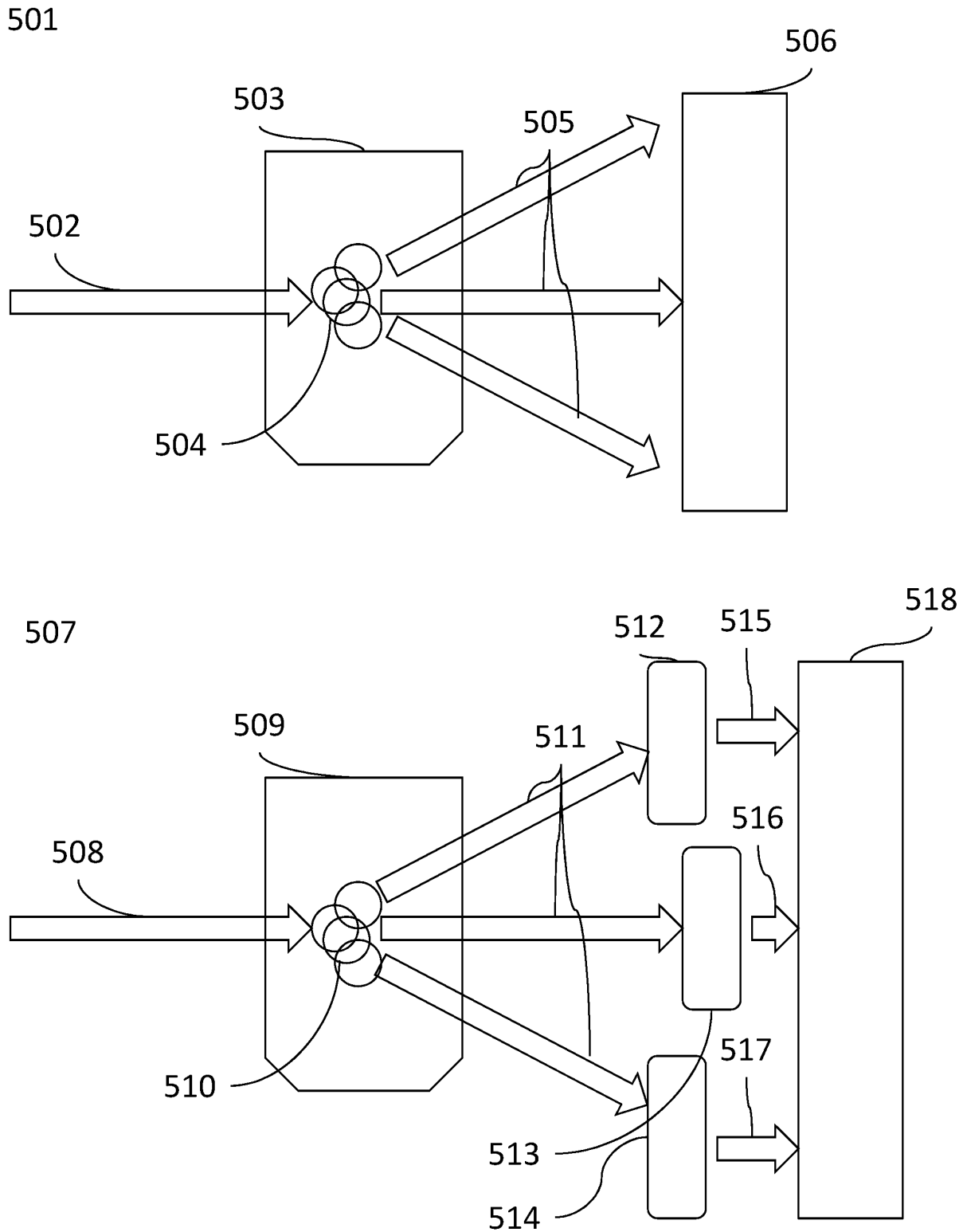
Fig. 5

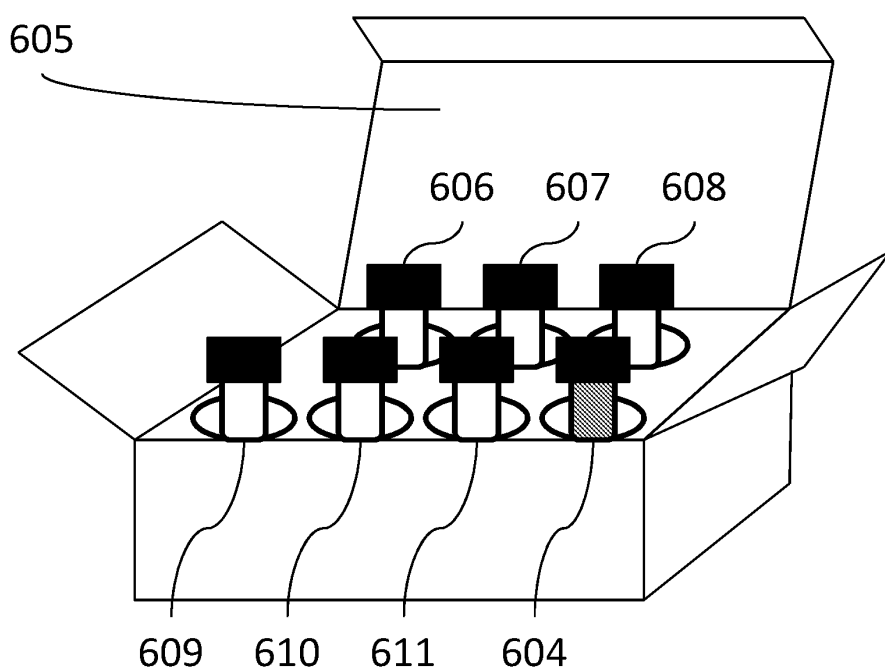
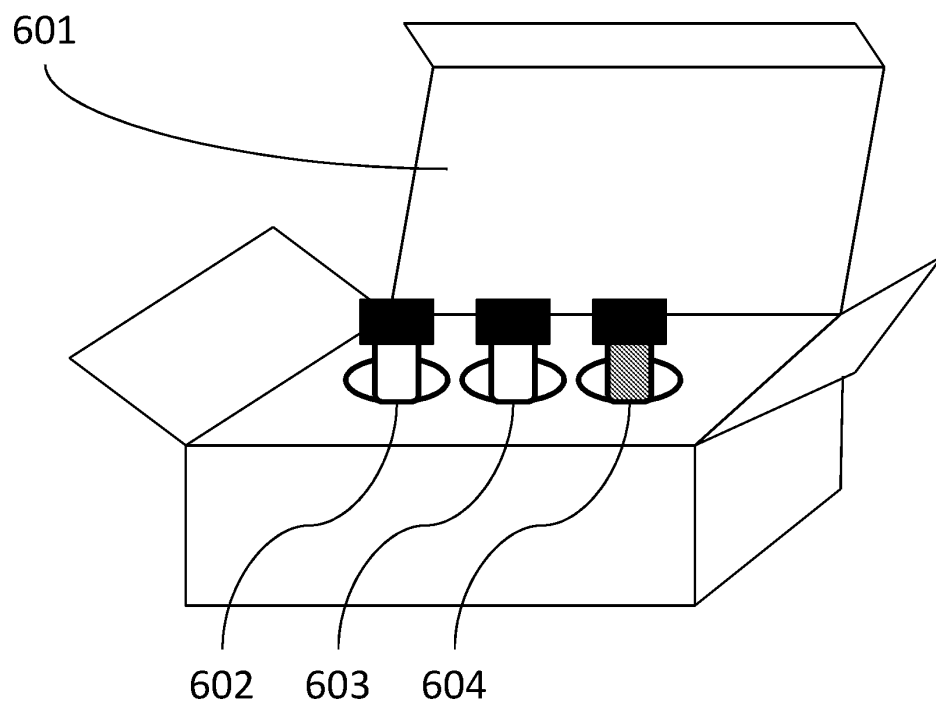
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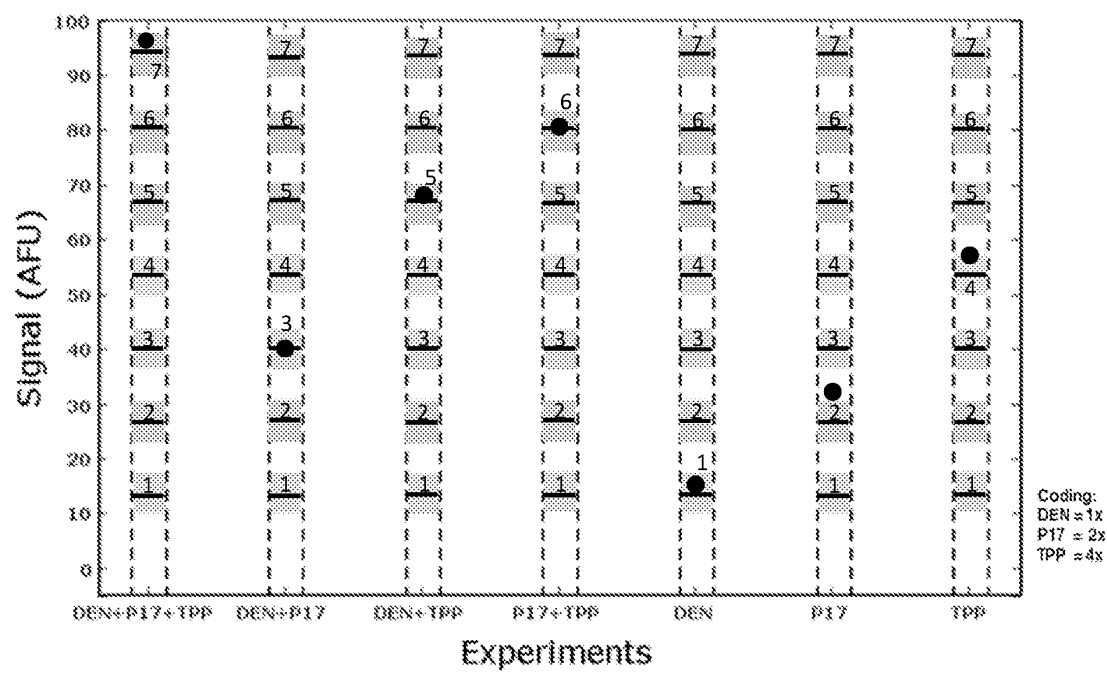
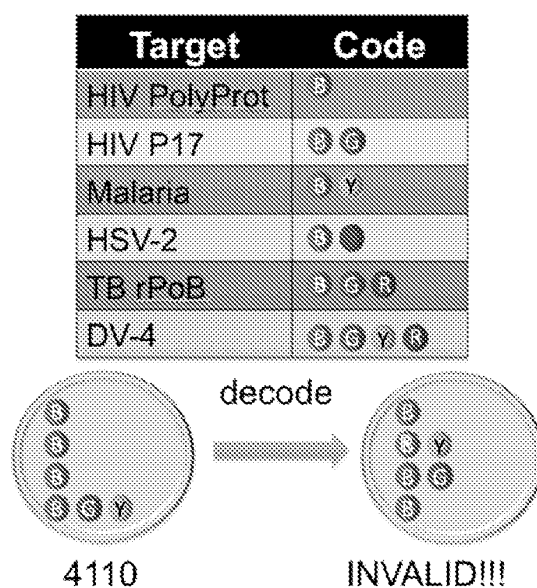
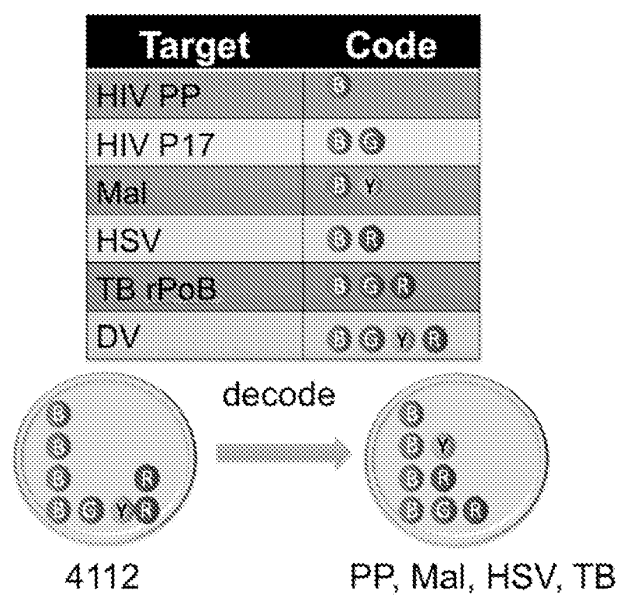
Fig. 7

Fig. 8

REQUEST FOR TRANSFER OF A COMPUTER READABLE FORM UNDER 37 CFR 1.821(e)			
Application No.:		First Named Inventor:	Emil P. Kartalov
Filing Date:	May 1, 2020	Practitioner Docket No.:	38075-716.304
Title of the Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS		
The sequence information in the paper copy or PDF file of the Sequence Listing submitted in this application in accordance with 37 CFR 1.821(c), ☒ as part of the originally-filed specification of this application ☐ as a separate amendment of the specification filed ☐ herewith ☐ on _____ is identical to the sequence information in the ☒ only computer readable form ☐ last filed computer readable form ☐ other computer readable form, which was filed on _____ in application number <u>14/451,876</u>, which was filed on <u>August 5, 2014</u>. This computer readable form was compliant with 37 CFR 1.821-1.825, and applicant hereby requests that it be used as the computer readable form for the present application, in accordance with 37 CFR 1.821(e).			
The above referenced paper copy or PDF file of the Sequence Listing contains no new matter.			
A sequence listing text file submitted via EFS-Web that complies with the requirements of 37 CFR 1.824(a) (2)-(6) and (b) (i.e., is a compliant sequence listing ASCII text file), serves as both the paper copy required by 37 CFR 1.821(c) and the CRF required by 37 CFR 1.821(e). If a user submits a compliant sequence listing ASCII text file via EFS-Web, the U.S. Patent and Trademark Office will not carry out a request to use a compliant computer readable "Sequence Listing" that is already on file for another application pursuant to 37 CFR 1.821(e) but will use the sequence listing submitted with the application as originally filed via EFS-Web.			
It is understood that upon the transfer of a copy of the computer readable form to this application, the U.S. Patent and Trademark Office will update the copy of the computer readable form to reflect the application number and filing date for this application.			
Signature /Nicholas Tiee/		Date <u>May 1, 2020</u>	
Name (Print/Typed) <u>Nicholas Tiee</u>		Practitioner Registration Number <u>77,907</u>	
Note: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required. *			
☒ *Total of <u>1</u> forms are submitted.			

This collection of information is required by 35 U.S.C. 22 and 37 CFR 1.821-1.825. The information is required to obtain or retain a benefit by the public, which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 6 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS.

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SEQUENCE LISTING

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RAJAGOPAL, ADITYA
SCHERER, AXEL

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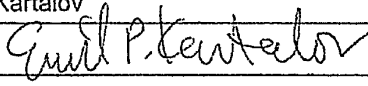
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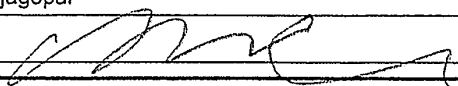
**DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN
APPLICATION DATA SHEET (37 CFR 1.76)**

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 <u>13/756,760</u> filed on <u>February 1, 2013</u> 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: <u>Emil Kartalov</u> Date (Optional) : _____ Signature: <u></u>	
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.

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

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

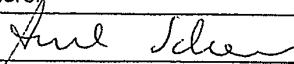
Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
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LEGAL NAME OF INVENTOR Inventor: <u>Aditya Rajagopal</u> Date (Optional): <u>02/28/2013</u> Signature: 	
Note: An application data sheet (PTO/AIA/14 or equivalent), including naming the entire inventive entity, must accompany this form. Use an additional PTO/SB/AIA01 form for each additional inventor.	

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.

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

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

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 <u>13/756,760</u> filed on <u>February 1, 2013</u>. 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: <u>Axel Scherer</u> Date (Optional): <u>2/28/13</u> Signature: <u></u> 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.

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

Electronic Acknowledgement Receipt	
EFS ID:	39328892
Application Number:	16864744
International Application Number:	
Confirmation Number:	9929
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil P. Kartalov
Customer Number:	21971
Filer:	Nicholas Shaofeng Tiee/Cecilia Hui
Filer Authorized By:	Nicholas Shaofeng Tiee
Attorney Docket Number:	38075-716.304
Receipt Date:	01-MAY-2020
Filing Date:	
Time Stamp:	15:46:08
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
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	Specification		1	68	
	Claims		69	72	
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Total Files Size (in bytes):	4069815
This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503. <u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application. <u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.	

RAW SEQUENCE LISTING

Loaded by SCORE, no errors detected.

Application Serial Number: 16864744

Source: OPAP

Date Processed by SCORE: 05/06/2020

ENTERED

16864744
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RAJAGOPAL, ADITYA
SCHERER, AXEL

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<130> 38075-716.301

<140> US 16/864,744

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United States Patent and Trademark Office
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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO	TOT CLAIMS	IND CLAIMS
16/864,744	05/01/2020		0.00	38075-716.304	21	5

CONFIRMATION NO. 9929

21971

WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

FILING RECEIPT



0000000117191885

Date Mailed: 05/22/2020

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please submit a written request for a corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections provided that the request is grantable.

Inventor(s)

Emil P. Kartalov, Pasadena, CA;
Aditya Rajagopal, Orange, CA;
Axel Scherer, Pasadena, CA;

Applicant(s)

California Institute of Technology, Pasadena, CA;

Power of Attorney: The patent practitioners associated with Customer Number 21971

Domestic Priority data as claimed by applicant

This application is a CON of 15/914,356 03/07/2018
which is a CON of 14/451,876 08/05/2014 PAT 10068051
which is a CON of 13/756,760 02/01/2013 PAT 8838394
which claims benefit of 61/703,093 09/19/2012
and claims benefit of 61/594,480 02/03/2012

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see <http://www.uspto.gov> for more information.) - None.

Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

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

If Required, Foreign Filing License Granted: 05/21/2020

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/864,744**

Projected Publication Date: To Be Determined - pending completion of Missing Parts

Non-Publication Request: No

Early Publication Request: No
Title

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Preliminary Class

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

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Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

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page 2 of 4

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Title 37, Code of Federal Regulations, 5.11 & 5.15

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/864,744	05/01/2020	Emil P. Kartalov	38075-716.304

CONFIRMATION NO. 9929

21971

WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

FORMALITIES LETTER



Date Mailed: 05/22/2020

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

Items Required To Avoid Abandonment:

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- The statutory basic filing fee is missing.
- The application search fee must be submitted.
- The application examination fee must be submitted.
- Additional claim fees of \$ **1020** as an undiscounted entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due.
- Surcharge as set forth in 37 CFR 1.16(f) must be submitted.
The surcharge is due for any one of:
 - late submission of the basic filing fee, search fee, or examination fee,
 - late submission of inventor's oath or declaration,
 - filing an application that does not contain at least one claim on filing, or
 - submission of an application filed by reference to a previously filed application.

SUMMARY OF FEES DUE:

The fee(s) required within **TWO MONTHS** from the date of this Notice to avoid abandonment is/are itemized below. No entity status discount is in effect. If applicant is qualified for small entity status, a written assertion of small entity status must be submitted to establish small entity status. (See 37 CFR 1.27). If applicant is qualified for micro entity status, an acceptable Certification of Micro Entity Status must be submitted to establish micro entity status. (See 37 CFR 1.29 and forms PTO/SB/15A and 15B.)

- \$ **300** basic filing fee.
- \$ **160** surcharge.
- \$ **660** search fee.
- \$ **760** examination fee.
- \$ **920** for **2** independent claims over 3.
- \$ **100** for **1** total claims over 20.

- \$(0) previous unapplied payment amount.
- **\$ 2900** TOTAL FEE BALANCE DUE.

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

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<https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html>

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Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at
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/fasrat/

Electronically filed on: December 16, 2020

PATENT

Attorney Docket No.: 38075-716.304

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventors: Emil Kartalov, *et al.*

Serial Number: 16/864,744

Filing Date: May 1, 2020

Title: SIGNAL ENCODING AND DECODING IN
MULTIPLEXED BIOCHEMICAL ASSAYS

Group Art Unit: Unassigned

Examiner: Unassigned

CONFIRMATION NO: 9929

**RESPONSE TO NOTICE TO FILE MISSING PARTS AND
PRELIMINARY AMENDMENT**

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313

Dear Commissioner:

This paper is filed in response to the Notice to File Missing Parts mailed May 22, 2020. Prior to an examination on the merits of the above-identified application, please amend the application as follows:

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

Remarks begin on page 4 of this paper.

AMENDMENTS TO THE CLAIMS

- 1.– 20. (Canceled)
21. (Original) An assay that is capable of unambiguously detecting the presence or absence of each of at least M analytes,
 wherein each of said M analytes is encoded as a value of one component of a fluorescent signal,
 wherein said fluorescent signal comprises F wavelength components, and
 wherein said assay is capable of detecting the presence or absence of each of said at least M analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis, based on detection of said F wavelength components, when M is greater than F.
22. (New) The assay of claim 21, wherein each of said at least M analytes is encoded as at least one first value in a first component of said fluorescent signal and at least one second value in a second component of said fluorescent signal.
23. (New) The assay of claim 22, wherein said first value is an intensity or range of intensities.
24. (New) The assay of claim 21, wherein said detecting is performed with reagents comprising hybridization probes.
25. (New) The assay of claim 24, wherein said hybridization probes comprise one or more hybridization probes specific for different analytes and comprising an identical fluorophore or combination of fluorophores.
26. (New) The assay of claim 21, wherein said signal is generated during a polymerase chain reaction.
27. (New) The assay of claim 21, wherein at least one of said analytes is encoded by at least one additional value, wherein said at least one additional value is selected from the group consisting of a value from at least one additional component of said signal, a value from at least one component of an additional signal, and combinations thereof.
28. (New) The assay of claim 21, wherein said fluorescence signal from said hybridization probes are used to construct a chromatogram.
29. (New) The assay of claim 21, wherein at least one of said analytes comprises a polynucleotide.
30. (New) The assay of claim 21, further comprising transmitting information concerning the presence or absence of said analyte through a computer network.

31. (New) The assay of claim 21, wherein at least one step of said assay is performed using a computer and wherein said computer is connected to a thermal cycler.
32. (New) The assay of claim 21, wherein said coding scheme is innately non-degenerate or non-degenerate by construction.
33. (New) The assay of claim 22, wherein said coding scheme is made non-degenerate by enumerating every legitimate result that can be obtained from said coding scheme, identifying each legitimate result that is degenerate, and eliminating at least one potential analyte code from said coding scheme to eliminate degeneracy
34. (New) A kit for detecting the presence or absence of at least M analytes by generating a single cumulative measurement, comprising analyte-specific reagents, wherein:
 - a. each of said at least M analytes is encoded as a value of one component of a fluorescent signal, thereby generating a coding scheme, wherein each of said at least M analytes is represented in said coding scheme by said value, and wherein said encoding is performed in a manner that eliminates degeneracy; and
 - b. each of said analyte-specific reagents, when contacted with a sample comprising its corresponding analyte, generates the first value.
35. (New) The kit of claim 34, wherein said kit is capable of unambiguously detecting the presence or absence of each analyte of the plurality of analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, or melting curve analysis.
36. (New) The kit of claim 34, wherein said kit is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, when M is greater than F.
37. (New) The kit of any of claims 34, wherein said analyte-specific reagents comprise hybridization probes.
38. (New) The kit of claim 37, wherein said hybridization probes comprise a fluorophore, and wherein said plurality of hybridization probes are specific for different analytes.
39. (New) The kit of claim 38, wherein said number of hybridization probes in the kit is greater than the number of the analytes of the plurality of analytes.
40. (New) The kit of any of claims 34, wherein the analyte-specific reagents are each present at a different amount in the kit.

REMARKS

Claims 1-21 were pending prior to the entry of these amendments. Claims 1-20 are canceled. Claims 22-40 are new. The new claims are fully supported by the application as filed. No new matter is added. Accordingly, upon entry of these amendments, claims 21-40 are pending.

CONCLUSION

Applicant respectfully requests entry of the abovementioned amendments prior to the examination of the present application. Should the Examiner have any questions, the Examiner is encouraged to contact the undersigned at (650) 849-3327.

The Commissioner is authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 23-2415 (Docket No. 38075-716.304).

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI

Date: December 16, 2020

By: Nicholas Tice/
Nicholas Tice, Reg. No. 77,907

650 Page Mill Road
Palo Alto, California 94304
Telephone No.: (512) 338-5444
Facsimile No.: (650) 493-6811
Customer No. 021971

Electronic Patent Application Fee Transmittal				
Application Number:		16864744		
Filing Date:		01-May-2020		
Title of Invention:		SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS		
First Named Inventor/Applicant Name:		Emil P. Kartalov		
Filer:		Nicholas Shaofeng Tiee/Cecilia Hui		
Attorney Docket Number:		38075-716.304		
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
UTILITY APPLICATION FILING	1011	1	320	320
UTILITY SEARCH FEE	1111	1	700	700
UTILITY EXAMINATION FEE	1311	1	800	800
Pages:				
Claims:				
Miscellaneous-Filing:				
LATE FILING FEE FOR OATH OR DECLARATION	1051	1	160	160
Petition:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 5 months with \$0 paid	1255	1	3160	3160
Miscellaneous:				
Total in USD (\$)				5140

Electronic Acknowledgement Receipt	
EFS ID:	41407142
Application Number:	16864744
International Application Number:	
Confirmation Number:	9929
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil P. Kartalov
Customer Number:	21971
Filer:	Nicholas Shaofeng Tiee/Cecilia Hui
Filer Authorized By:	Nicholas Shaofeng Tiee
Attorney Docket Number:	38075-716.304
Receipt Date:	16-DEC-2020
Filing Date:	01-MAY-2020
Time Stamp:	17:56:13
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$5140
RAM confirmation Number	E2020BFH57140797
Deposit Account	232415
Authorized User	Cecilia Hui
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	Power of Attorney	38075_716304_POA.pdf	158238	no	2
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Warnings:					
Information:					
2		38075_716304_PA.pdf	150208	yes	5
			ba0ee40a87c0354a66f125d1ffa965924664f87		
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Preliminary Amendment		1	1	
	Claims		2	3	
	Applicant Arguments/Remarks Made in an Amendment		4	5	
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Total Files Size (in bytes):			347476		

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date
16/864,744	May 1, 2020

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

☒ I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above:

021971

OR

☐ I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:

☒ The address associated with the above-mentioned Customer Number

OR

☐ The address associated with Customer Number:

OR

☐ Firm or Individual Name

Address

City

State

Zip

Country

Telephone

Email

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

- ☐ Inventor or Joint Inventor (title not required below)
- ☐ Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)
- ☒ Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)
- ☐ Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature /Fred Farina/

Date (Optional) 2020-05-12

Name Fred Farina

Title Chief Innovation and Corporate Partnerships Officer, California Institute of Technology

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

☒ Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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

Doc Code: PA..

Document Description: Power of Attorney

PTO/AIA/82A (07-13)

Approved for use through 03/31/2021. OMB 0651-0035

U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TRANSMITTAL FOR POWER OF ATTORNEY TO ONE OR MORE REGISTERED PRACTITIONERS

NOTE: This form is to be submitted with the Power of Attorney by Applicant form (PTO/AIA/82B) to identify the application to which the Power of Attorney is directed, in accordance with 37 CFR 1.5, unless the application number and filing date are identified in the Power of Attorney by Applicant form. If neither form PTO/AIA/82A nor form PTO/AIA/82B identifies the application to which the Power of Attorney is directed, the Power of Attorney will not be recognized in the application.

Application Number	16/864,744
Filing Date	May 1, 2020
First Named Inventor	Emil P. Kartalov
Title	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
Art Unit	Unassigned
Examiner Name	Unassigned
Attorney Docket Number	38075-716.304

SIGNATURE of Applicant or Patent Practitioner

Signature	/Nicholas Tiee/	Date (Optional)	2020-12-16
Name	Nicholas Tiee	Registration Number	77,907
Title (if Applicant is a juristic entity)			
Applicant Name (if Applicant is a juristic entity)			

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. If more than one applicant, use multiple forms.



*Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875						Application or Docket Number 16/864,744				
APPLICATION AS FILED - PART I										
(Column 1)		(Column 2)		SMALL ENTITY		OTHER THAN SMALL ENTITY				
FOR	NUMBER FILED	NUMBER EXTRA	RATE(\$)	FEE(\$)		RATE(\$)	FEE(\$)			
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	320			
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A			N/A	700			
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	800			
TOTAL CLAIMS (37 CFR 1.16(j))	20	minus 20 = *			OR	x 100 =	0.00			
INDEPENDENT CLAIMS (37 CFR 1.16(h))	2	minus 3 = *			OR	x 480 =	0.00			
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$310 (\$155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00			
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00			
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL		TOTAL	1820			
APPLICATION AS AMENDED - PART II										
(Column 1)		(Column 2)		(Column 3)		SMALL ENTITY		OTHER THAN SMALL ENTITY		
AMENDMENT A		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE(\$)	ADDITIONAL FEE(\$)		RATE(\$)	ADDITIONAL FEE(\$)
	Total (37 CFR 1.16(i))	*	Minus	**	=	x	=	OR	x	=
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x	=	OR	x	=
	Application Size Fee (37 CFR 1.16(s))							OR		
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
TOTAL ADD'L FEE								OR	TOTAL ADD'L FEE	
AMENDMENT B		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE(\$)	ADDITIONAL FEE(\$)		RATE(\$)	ADDITIONAL FEE(\$)
	Total (37 CFR 1.16(i))	*	Minus	**	=	x	=	OR	x	=
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x	=	OR	x	=
	Application Size Fee (37 CFR 1.16(s))							OR		
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
TOTAL ADD'L FEE								OR	TOTAL ADD'L FEE	
* If the entry in column 1 is less than the entry in column 2, write "0" in column 3. ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3". The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.										



UNITED STATES PATENT AND TRADEMARK OFFICE

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

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO	TOT CLAIMS	IND CLAIMS
16/864,744	05/01/2020		1980	38075-716.304	20	2

CONFIRMATION NO. 9929

UPDATED FILING RECEIPT

21971

WILSON SONSINI GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050



0000000122111789

Date Mailed: 12/22/2020

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please submit a written request for a corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections provided that the request is grantable.

Inventor(s)

Emil P. Kartalov, Pasadena, CA;
Aditya Rajagopal, Orange, CA;
Axel Scherer, Pasadena, CA;

Applicant(s)

California Institute of Technology, Pasadena, CA;

Power of Attorney: The patent practitioners associated with Customer Number 21971

Domestic Priority data as claimed by applicant

This application is a CON of 15/914,356 03/07/2018 PAT 10770170
which is a CON of 14/451,876 08/05/2014 PAT 10068051
which is a CON of 13/756,760 02/01/2013 PAT 8838394
which claims benefit of 61/703,093 09/19/2012
and claims benefit of 61/594,480 02/03/2012

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see <http://www.uspto.gov> for more information.) - None.

Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

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

If Required, Foreign Filing License Granted: 05/21/2020

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/864,744**

Projected Publication Date: 04/01/2021

Non-Publication Request: No

Early Publication Request: No

Title

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Preliminary Class

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

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at <http://www.uspto.gov/web/offices/pac/doc/general/index.html>.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, <http://www.stopfakes.gov>. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific

page 2 of 4

countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER
Title 35, United States Code, Section 184
Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign Assets Control, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

SelectUSA

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop

technology, manufacture products, deliver services, and grow your business, visit <http://www.SelectUSA.gov> or call +1-202-482-6800.



UNITED STATES PATENT AND TRADEMARK OFFICE

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

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/864,744	05/01/2020	Emil P. Kartalov	38075-716.304

CONFIRMATION NO. 9929

POA ACCEPTANCE LETTER

21971
WILSON SONSINI GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050



Date Mailed: 12/22/2020

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 12/16/2020.

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

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

/Itaba/

PATENT COOPERATION TREATY



From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT AND
THE WRITTEN OPINION OF THE INTERNATIONAL
SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

To: NICHOLAS TIEE WILSON SONSINI GOODRICH & ROSATI 650 PAGE MILL ROAD PALO ALTO, CA 94304		Date of mailing (day/month/year) 21 NOV 2019
Applicant's or agent's file reference 44158-711.601	FOR FURTHER ACTION See paragraphs 1 and 4 below	
International application No. PCT/US2019/050050	International filing date (day/month/year) 06 September 2019	
Applicant CHROMACODE, INC.		

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

Filing of amendments and statement under Article 19:
 The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

How? Directly to the International Bureau of WIPO preferably through ePCT or on paper to, 34 chemin des Colombettes
 1211 Geneva 20, Switzerland, Facsimile No.: +41 22 338 82 70

For more detailed instructions, see *PCT Applicant's Guide*, International Phase, paragraphs 9.004 – 9.011.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. ☐ **With regard to any protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:**

☐ the protest together with the decision thereon has been transmitted to the International Bureau together with any request to forward the texts of both the protest and the decision thereon to the designated Offices.

☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. These comments will be made available to the public after international publication. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established.

Shortly after the expiration of **18 months from the priority date, the international application will be published** by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before the completion of the technical preparations for international publication (Rules 90bis.1 and 90bis.3).

Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for **entry into the national phase** before those designated Offices. In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months. For details about the applicable time limits, Office by Office, see www.wipo.int/pct/en/texts/time_limits.html and the *PCT Applicant's Guide*, National Chapters.

Within **22 months** from the priority date, the applicant may request that a **supplementary international search** be carried out by a different International Searching Authority that offers this service (Rule 45bis.1). The procedure for requesting supplementary international search is described in the *PCT Applicant's Guide*, International Phase, paragraphs 8.006-8.032.

Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 Telephone No. PCT OSP: 571-272-7774
---	--

Form PCT/ISA/220 (July 2017)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 44158-711.601	FOR FURTHER ACTION see Form PCT/ISA/220 as well as, where applicable, item 5 below.	
International application No. PCT/US2019/050050	International filing date (<i>day/month/year</i>) 06 September 2019	(Earliest) Priority Date (<i>day/month/year</i>) 07 September 2018
Applicant CHROMACODE, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the **language**, the international search was carried out on the basis of:

- ☒ the international application in the language in which it was filed.
☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6bis(a)).

c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☒ **Certain claims were found unsearchable** (see Box No. II).

3. ☐ **Unity of invention is lacking** (see Box No. III).

4. With regard to the **title**,

- ☒ the text is approved as submitted by the applicant.
☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

- ☒ the text is approved as submitted by the applicant.
☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the **drawings**,

- a. the figure of the **drawings** to be published with the abstract is Figure No. 1
☒ as suggested by the applicant.
☐ as selected by this Authority, because the applicant failed to suggest a figure.
☐ as selected by this Authority, because this figure better characterizes the invention.
- b. ☐ none of the figures is to be published with the abstract.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/050050

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.:
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:

3. ☒ Claims Nos.: 24, 103
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 additional 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 additional fees, this Authority did not invite payment of additional fees.
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.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/050050

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07H 21/04; C12Q 1/68; C12Q 1/6848; C12Q 1/6858; C12Q 1/686; G01N 21/64 (2019.01)
CPC - B01J 2219/00659; C07H 21/04; C12Q 1/6818; C12Q 1/6827; C12Q 1/6848; C12Q 1/6858; C12Q 1/686; C12Q 2525/161; C12Q 2565/1025; G01N 2021/6417 (2019.08)

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)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/139.1; 424/158.1; 435/6.11; 435/287.2; 506/9 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2014/0274774 A1 (LIFE TECHNOLOGIES CORPORATION) 18 September 2014 (18.09.2014) entire document	1-23, 25-47, 49-68, 70-75, 77-79, 81-102, 104-112, 115-126, 128-147, 149-154
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Y		48, 69, 76, 80, 113, 114, 127, 148
Y	WO 2001/007640 A2 (THE SECRETARY OF STATE FOR THE HOME DEPARTMENT et al) 01 February 2001 (01.02.2001) entire document	48, 113, 114, 127
Y	US 2017/0362636 A1 (CALIFORNIA INSTITUTE OF TECHNOLOGY) 21 December 2017 (21.12.2017) entire document	69, 76, 80, 148
A	US 2018/0057864 A1 (CHROMACODE, INC.) 01 March 2018 (01.03.2018) entire document	1-23, 25-102, 104-154
A	US 2018/0030551 A1 (CALIFORNIA INSTITUTE OF TECHNOLOGY) 01 February 2018 (01.02.2018) entire document	1-23, 25-102, 104-154
A	US 2016/0040256 A1 (LIFE TECHNOLOGIES CORPORATION) 11 February 2016 (11.02.2016) entire document	1-23, 25-102, 104-154
A	✓ NAVARRO et al. "Real-time PCR Detection Chemistry," Clinica Chimica Acta, 22 October 2014 (22.10.2014), Vol. 439, Pgs. 231-250. entire document	1-23, 25-102, 104-154

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
"E" earlier application or patent but published on or after the international filing date	"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
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"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	

Date of the actual completion of the international search
30 October 2019

Date of mailing of the international search report
21 NOV 2019

Name and mailing address of the ISA/US
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PCT OSP: 571-272-7774

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: NICHOLAS TIEE
WILSON SONSINI GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)

Date of mailing
(day/month/year)

21 NOV 2019

Applicant's or agent's file reference
44158-711.601

FOR FURTHER ACTION

See paragraph 2 below

International application No.

PCT/US2019/050050

International filing date (day/month/year)

06 September 2019

Priority date (day/month/year)

07 September 2018

International Patent Classification (IPC) or both national classification and IPC

IPC(8) - C07H 21/04; C12Q 1/68; C12Q 1/6848; C12Q 1/6858; C12Q 1/686; G01N 21/64 (2019.01)

CPC - B01J 2219/00659; C07H 21/04; C12Q 1/6818; C12Q 1/6827; C12Q 1/6848; C12Q 1/6858; C12Q 1/686; C12Q 2525/161; C12Q 2565/1025; G01N 2021/6417 (2019.08)

Applicant **CHROMACODE, INC.**

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450
Facsimile No. 571-273-8300

Date of completion of this opinion

30 October 2019

Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (January 2015)

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/US2019/050050

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:
 - ☒ the international application in the language in which it was filed.
 - ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).
2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a)).
3. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:
 - a. ☐ forming part of the international application as filed:
 - ☐ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).
4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
5. Additional comments:

**WRITTEN OPINION OF THE
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Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 24, 103

because:

☐ the said international application, or the said claims Nos. _____ relate to the following subject matter which does not require an international search (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 24, 103 are so unclear that no meaningful opinion could be formed (*specify*):

Claims 24 and 103 are multiple dependent claims not drafted in accordance with the second and third sentences of Rule 6.4(a).

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed (*specify*):

☒ no international search report has been established for said claims Nos. 24, 103

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

☐ furnish a sequence listing in the form of an Annex C/ST.25 text file, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.

☐ furnish a sequence listing on paper or in the form of an image file complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.

☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13ter.1(a) or (b).

☐ See Supplemental Box for further details.

**WRITTEN OPINION OF THE
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Box No. V	Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement		
1. Statement			
Novelty (N)	Claims	see claims below	YES
	Claims	see claims below	NO
Inventive step (IS)	Claims	None	YES
	Claims	1-23, 25-102, 104-154	NO
Industrial applicability (IA)	Claims	1-23, 25-102, 104-154	YES
	Claims	None	NO
2. Citations and explanations:			
Novelty (N) YES: 48, 69, 76, 80, 113, 114, 127, 148 NO: 1-23, 25-47, 49-68, 70-75, 77-79, 81-102, 104-112, 115-126, 128-147, 149-154 Claims 1-23, 25-47, 49-68, 70-75, 77-79, 81-102, 104-112, 115-126, 128-147, and 149-154 lack novelty under PCT Article 33(2) as being anticipated by US 2014/0274774 A1 to Life Technologies Corporation (hereinafter Life Technologies). Regarding Claim 1, Life Technologies discloses a method for determining the presence or absence of a target nucleic acid in the presence of a non-target nucleic acid (Abstract, The present disclosure is drawn to methods for detection, quantitation and analysis of nucleotides of interest, for example SNPs, in nucleic acid sequences of interest using universal FRET-based reporter primers), said method comprising: (A) providing a sample comprising, or potentially comprising, said target nucleic acid and said non-target nucleic acid (Para. [0076], The target nucleic acid may be obtained from any Source, and may comprise any number of different compositional components.... target nucleic acids may be isolated from Samples using any of a variety of procedures known in the art); (B) forming a mixture comprising: i. said sample; ii. a forward primer comprising a first region configured to hybridize to said target nucleic acid under amplification conditions and configured to not hybridize to said non-target nucleic acid under said amplification conditions, and a second region configured to not hybridize to said target nucleic acid under said amplification conditions; and iii. a signal generating nucleic acid probe (Para. [0007], oligonucleotides are provided that are specific for a particular allele or polymorphism. Such oligonucleotides are referred to herein as allele-specific primers or ASPs. Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1). In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0021], the composition and/or reaction mixture includes one or more target nucleic acid molecules and a first allele-specific primer, which is specific for a first allele, including a first 5'-universal tail which includes a binding site for a first UFP, wherein the first UFP includes a first fluorophore and at least a first quencher moiety), wherein said signal generating nucleic acid probe anneals to said second region, or regions complementary thereto, when subjected to said amplification conditions (Para. [0007], the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1). In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer); (C) subjecting said mixture to said amplification conditions, said amplification conditions appropriate to amplify said target nucleic acid with an amplification reaction, thereby amplifying said target nucleic acid such that said signal generating nucleic acid probe is degraded and a signal is generated if said target nucleic acid is present in said mixture (Para. [0012], oligonucleotides are provided that may be used to detect the amplification of a target nucleic acid and/or may be used to monitor or detect the amplification of an allele or polymorphism in a target nucleic acid. Such oligonucleotides are referred to herein as "universal FRET-based detector probes" the fluorescence may be further increased when the probe is digested by 5'-nuclease activity of polymerase; Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein); and (D) detecting the presence or absence of said signal, thereby determining the presence or absence of said target nucleic acid in the presence of said non-target nucleic acid (Para. [0115], TaqMan R assays are typically carried out by performing nucleic acid amplification on a target polynucleotide using a nucleic acid polymerase having 5'-to-3' nuclease activity, a primer capable of hybridizing to the target polynucleotide, and an oligonucleotide probe.... Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification)).			

Form PCT/ISA/237 (Box No. V) (January 2015)

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/US2019/050050

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 69, 149, and 153 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because claims 69, 149, and 153 are indefinite for the following reason(s):

Regarding claims 69 and 148, these claims recite the phrase "said second universal probe binding motif and said universal binding motif are on the 3' end of said first region and are on the 5' of said universal primer binding motif," which is inconsistent with the parent claim (claim 1). Specifically, these claims place the universal sequences at the 3' end of the primer and the target specific sequences of the first region at the 5' end, which is inconsistent with the sequence functioning as a target specific amplification primer as specified in the parent claim. For purposes of this written opinion, the phrase "said second universal probe binding motif and said universal binding motif are on the 3' end of said first region and are on the 5' of said universal primer binding motif" is best interpreted to read "said second universal probe binding motif and said first universal probe binding motif are on the 5' end of said first region and are on the 3' end of said universal primer binding motif" to be consistent with the universal tailed primer recited in claim 1, and the instant disclosure (see Para. [0040], and Fig. 6 of the instant application).

Regarding claim 153, the claim states "said sample" and there is no antecedent basis for a sample. For purposes of this written opinion, claim 153 is best interpreted to read "a sample".

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

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PCT/US2019/050050

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

Regarding Claim 2, Life Technologies discloses the method of claim 1, wherein said target nucleic acid is an allele (Para. [0007], oligonucleotides are provided that are specific for a particular allele or polymorphism. Such oligonucleotides are referred to herein as allele-specific primers or ASPs. Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer).

Regarding Claim 3, Life Technologies discloses the method of claim 1, wherein said target nucleic acid is a mutant sequence and said non-target nucleic acid is a wild type sequence (Para. [0151], The methods described herein may be also be used to detect rare alleles in the presence of wild type alleles (e.g., one mutant allele in the presence of 10³-10⁴ wild type alleles)).

Regarding Claim 4, Life Technologies discloses the method of claim 1, wherein the sequence of said target nucleic acid and the sequence of said non-target nucleic acid is different at least one nucleotide (Para. [0006], Provided herein are oligonucleotides, compositions, reaction mixtures, methods and kits for genotyping analysis, including, but not limited to SNP genotyping analysis; Para. [0083], As used herein, the term "single nucleotide polymorphism" (SNP) refers to a DNA sequence variation occurring when a single nucleotide, e.g., A, T, C, or G, in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA contain a difference in a single nucleotide).

Regarding Claim 5, Life Technologies discloses the method of claim 1, wherein the sequence of said target nucleic acid and the sequence of said non-target nucleic acid is different by only one nucleotide (Para. [0006], Provided herein are oligonucleotides, compositions, reaction mixtures, methods and kits for genotyping analysis, including, but not limited to SNP genotyping analysis; Para. [0083], As used herein, the term "single nucleotide polymorphism" (SNP) refers to a DNA sequence variation occurring when a single nucleotide, e.g., A, T, C, or G, in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA contain a difference in a single nucleotide).

Regarding Claim 6, Life Technologies discloses the method of claim 1, wherein the sequence of said target nucleic acid and the sequence of said non-target nucleic acid is different by no more than five nucleotides (Para. [0006], Provided herein are oligonucleotides, compositions, reaction mixtures, methods and kits for genotyping analysis, including, but not limited to SNP genotyping analysis; Para. [0083], As used herein, the term "single nucleotide polymorphism" (SNP) refers to a DNA sequence variation occurring when a single nucleotide, e.g., A, T, C, or G, in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA contain a difference in a single nucleotide).

Regarding Claim 7, Life Technologies discloses the method of claim 1, wherein said forward primer is specific to and selectively amplifies only said target nucleic acid (Para. [0056], The term "selectively hybridize" and variations thereof, means that, under appropriate stringency conditions, a given sequence (for example, but not limited to, a primer or probe) anneals with a second sequence comprising a complementary String of nucleotides (for example, but not limited to, a target flanking sequence or primer binding site of an amplicon), but does not anneal to undesired sequences, such as non-target nucleic acids, probes, or other primers; Para. [0130], the desired nucleic acid strand of the target allele-specific DNA synthesized on the template strand is amplified preferentially over any nucleic acid strand synthesized on the non-target allele-specific DNA).

Regarding Claim 8, Life Technologies discloses the method of claim 1, wherein said mixture does not employ the use of a nucleic acid or peptide blocking agents (Para. [0007], In certain embodiments (only in certain embodiments), the 3' end of the allele-specific primer includes a blocking agent such that the blocked end may be activated by a pyro phosphorolysis enzyme. In certain embodiments, the 3'-end of the allele-specific primer is extendable(not blocked) by a nucleic acid polymerase enzyme).

Regarding Claim 9, Life Technologies discloses the method of claim 1, wherein said mixture further comprising a reverse primer (Para. [0007], In certain embodiments, oligonucleotides are provided that are specific for a particular locus of the target nucleic acid. Such oligonucleotides are referred to herein as locus-specific primers or LSPs. In certain embodiments, the locus-specific primer may serve as a reverse primer in the amplification and genotyping methods disclosed herein).

Regarding Claim 10, Life Technologies discloses the method of claim 1, wherein said forward primer is configured to hybridize to a first area of a nucleic acid sequence of said target nucleic acid, and said reverse primer is configured to hybridize to a second area of the nucleic acid sequence of said target nucleic acid, thereby being configured to amplify the nucleic acid sequence under conditions sufficient for nucleic acid amplification (Para. [0007], In certain embodiments, the 3'-end of the allele-specific primer is extendable by a nucleic acid polymerase enzyme....In certain embodiments, oligonucleotides are provided that are specific for a particular locus of the target nucleic acid. Such oligonucleotides are referred to herein as locus-specific primers or LSPs. In certain embodiments, the locus-specific primer may serve as a reverse primer in the amplification and genotyping methods disclosed herein).

Regarding Claim 11, Life Technologies discloses the method of claim 10, wherein said first area comprises a 3' end of said nucleic acid sequence (Fig. 1, depicting the allele specific primer extending from the 3' end of the target and, and the locus specific or reverse primer extending from the opposite end (i.e. 5' end) of the target).

Regarding Claim 12, Life Technologies discloses the method of claim 10, wherein said second area comprises a 5' end of said nucleic acid sequence (Fig. 1, depicting the allele specific primer extending from the 3' end of the target and, and the locus specific or reverse primer extending from the opposite end (i.e. 5' end) of the target).

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/US2019/050050

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

Regarding Claim 13, Life Technologies discloses the method of claim 9, wherein said reverse primer comprises a sequence with complementarity or is homologous to a sequence on both target nucleic acid and nontarget nucleic acid (Para. [0068]). According to certain embodiments, the amplification of nucleic acid sequences may involve the use of locus-specific, group-specific, and/or allele-specific primers. Locus-specific primers (LSPs) may be used to amplify all alleles encoded at a given locus).

Regarding Claim 14, Life Technologies discloses the method of claim 9, wherein said reverse primer is a locus specific primer (Para. [0007]). In certain embodiments, the 3'-end of the allele-specific primer is extendable by a nucleic acid polymerase enzyme. In certain embodiments, oligonucleotides are provided that are specific for a particular locus of the target nucleic acid. Such oligonucleotides are referred to herein as locus-specific primers or LSPs. In certain embodiments, the locus-specific primer may serve as a reverse primer in the amplification and genotyping methods disclosed herein).

Regarding Claim 15, Life Technologies discloses the method of claim 9, wherein said reverse primer is a universal primer (Para. [0068]). According to certain embodiments, the amplification of nucleic acid sequences may involve the use of locus-specific, group-specific, and/or allele-specific primers. Locus-specific primers (LSPs) may be used to amplify all alleles encoded at a given locus (i.e. the primer is universal for all the alleles of a locus)).

Regarding Claim 16, Life Technologies discloses the method of claim 9, wherein said reverse primer is configured such that upon said thermally cycling said signal generating nucleic acid probe is digested (Para. [0012]), oligonucleotides are provided that may be used to detect the amplification of a target nucleic acid and/or may be used to monitor or detect the amplification of an allele or polymorphism in a target nucleic acid. Such oligonucleotides are referred to herein as "universal FRET-based detector probes" the fluorescence may be further increased when the probe is digested by 5'-nuclease activity of polymerase; Para. [0115]. Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification); Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein).

Regarding Claim 17, Life Technologies discloses the method of claim 1, wherein said mixture further comprises a nucleic acid enzyme (Para. [0012]), oligonucleotides are provided that may be used to detect the amplification of a target nucleic acid and/or may be used to monitor or detect the amplification of an allele or polymorphism in a target nucleic acid. Such oligonucleotides are referred to herein as "universal FRET-based detector probes" the fluorescence may be further increased when the probe is digested by 5'-nuclease activity of polymerase; Para. [0115]. Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification); Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein).

Regarding Claim 18, Life Technologies discloses the method of claim 1, wherein said amplification reaction is a polymerase chain reaction (PCR) (Para. [0012]), oligonucleotides are provided that may be used to detect the amplification of a target nucleic acid and/or may be used to monitor or detect the amplification of an allele or polymorphism in a target nucleic acid. Such oligonucleotides are referred to herein as "universal FRET-based detector probes" the fluorescence may be further increased when the probe is digested by 5'-nuclease activity of polymerase; Para. [0115]. Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification); Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein).

Regarding Claim 19, Life Technologies discloses the method of claim 18, wherein said PCR is a quantitative polymerase chain reaction (qPCR) (Para. [0012]), oligonucleotides are provided that may be used to detect the amplification of a target nucleic acid and/or may be used to monitor or detect the amplification of an allele or polymorphism in a target nucleic acid. Such oligonucleotides are referred to herein as "universal FRET-based detector probes" the fluorescence may be further increased when the probe is digested by 5'-nuclease activity of polymerase; Para. [0115]. Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification); Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein).

Regarding Claim 20, Life Technologies discloses the method of claim 1, wherein said amplification conditions comprises: a dNTP, a salt, a buffer, or a combination thereof (Para. [0159]). In some embodiments, multiple primer sets are included. In one embodiment, the kit may include one or more of, for example, a buffer (e.g., Tris), one or more salts (e.g., KCl), glycerol, dNTPs (dA, dT, dG, dC, du), recombinant BSA (bovine serum albumin)).

Regarding Claim 21, Life Technologies discloses the method of claim 1, wherein said signal generating nucleic acid probe further comprises a signal tag (Para. [0115]). Typically, the detectable label and quencher molecule are part of a single probe; Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein).

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/US2019/050050

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

Regarding Claim 22, Life Technologies discloses the method of claim 21, wherein said signal tag generates said signal (Para. [0115]). Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification); Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein).

Regarding Claim 23, Life Technologies discloses the method of claim 22, wherein said signal tag generates said signal upon degradation of said cleavable, signal generating nucleic acid probe by the 5' to 3' exonuclease activity of said nucleic acid enzyme, thereby liberating said signal for detection by a real time PCR instrument (Para. [0084]: a reaction vessel comprises a well of a multi-well plate, a spot on a glass slide, or a channel or chamber of a microfluidics device, including without limitation a TaqMan(R) Low Density Array or a TaqMan (R) Open Array Real-Time PCR plate; Para. [0115]). Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification); Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein).

Regarding Claim 25, Life Technologies discloses the method of claim 1, wherein said second region comprises: (A) a target-specific tail segment; and (B) a universal tail segment (Para. [0007]). Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1)).

Regarding Claim 26, Life Technologies discloses the method of claim 25, wherein said first region is positioned on the 3' end of forward primer (Para. [0007]). Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1)....the 3'-end of the allele-specific primer is extendable by a nucleic acid polymerase enzyme).

Regarding Claim 27, Life Technologies discloses the method of claim 25, wherein said universal tail segment is located on the 5' end of said forward primer (Para. [0007]). Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1)....the 3'-end of the allele-specific primer is extendable by a nucleic acid polymerase enzyme).

Regarding Claim 28, Life Technologies discloses the method of claim 25, wherein said target-specific tail segment is flanked on the 3' by said target-specific segment, and flanked on the 5' end by said universal tail segment on said forward primer (Para. [0007]). Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1)....the 3'-end of the allele-specific primer is extendable by a nucleic acid polymerase enzyme; Para. [0056]. The term "selectively hybridize" and variations thereof, means that, under appropriate stringency conditions, a given sequence (for example, but not limited to, a primer or probe) anneals with a second sequence comprising a complementary String of nucleotides (for example, but not limited to, a target flanking sequence or primer binding site of an amplicon), but does not anneal to undesired sequences, such as non-target nucleic acids, probes, or other primers).

Regarding Claim 29, Life Technologies discloses the method of claim 25, wherein a plurality of forward primers is used, wherein each second region of said forward primers comprises identical nucleotide sequences (Para. [0052]). In certain embodiments, an amplification reaction comprises multiplex amplification, in which a multiplicity of different target nucleic acids and/or a multiplicity of different amplification product species are simultaneously amplified using a multiplicity of different primer sets; Para. [0098], oligonucleotides are provided that are specific for a particular allele or polymorphism. Such oligonucleotides are referred to herein as allele-specific primers or ASPs. Such allele-specific primers comprise an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail comprises a binding site for a universal FRET-based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1). In certain embodiments, the 5'-universal tail comprises a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail comprises a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer).

Regarding Claim 30, Life Technologies discloses the method of claim 25, wherein a plurality of forward primers is used, wherein each second region of said forward primers comprises dissimilar nucleotide sequences (Para. [0013]), a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP).

Regarding Claim 31, Life Technologies discloses the method of claim 25, wherein a plurality of forward primers is used, wherein each second region of said forward primers comprises unique nucleotide sequences (Para. [0013]), a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP; Para. [0052], a multiplicity of different amplification product species are simultaneously amplified using a multiplicity of different primer sets; Para. [0052], an amplification reaction comprises multiplex amplification, in which a multiplicity of different target nucleic acids and/or a multiplicity of different amplification product species are simultaneously amplified using a multiplicity of different primer sets; Para. [0069], each allele-specific primer may independently comprise a nucleotide sequence comprising a 5'-universal tail; Para. [0106]. An exemplary SNP assay where at least one universal FRET-based detector probe replaces the UFP is shown in FIG. 9. An assay may include a pair of allele-specific primers where each allele specific includes a universal tail (ASP1 and ASP2), a shared locus-specific reverse primer (LSP), and a pair of universal detector probes for each of the two alleles (i.e. a unique probe for each allele)).

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Regarding Claim 32, Life Technologies discloses the method of claim 1, wherein said signal generating nucleic acid probe comprises sequence complementary or homologous to said forward primer (Para. [0007]. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer).

Regarding Claim 33, Life Technologies discloses the method of claim 1, wherein said signal generating nucleic acid probe is a target specific probe (Para. [0106]. An exemplary SNP assay where at least one universal FRET-based detector probe replaces the UFP is shown in FIG. 9. An assay may include a pair of allele-specific primers where each allele specific includes a universal tail (ASP1 and ASP2), a shared locus-specific reverse primer (LSP), and a pair of universal detector probes for each of the two alleles (i.e. a unique probe for each allele)).

Regarding Claim 34, Life Technologies discloses the method of claim 25, wherein said signal generating nucleic acid probe is a target-specific probe comprising: (A) a sequence complementary or homologous to said 3' end of said universal tail; (B) a sequence complementary or homologous to said entire target-specific tail; and (C) a sequence complementary or homologous to a portion of said first region (Para. [0007]. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer).

Regarding Claim 35, Life Technologies discloses the method of claim 25, wherein said signal generating nucleic acid probe is a target-specific probe comprising: (A) a sequence complementary or homologous to said 3' end of said universal tail; and (B) a sequence complementary or homologous to a portion of the target-specific tail (Para. [0007]. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer).

Regarding Claim 36, Life Technologies discloses the method of claim 1, wherein said probe binds to said target nucleic acid in an amplicon generated by second strand synthesis initiated in said amplification reaction (Para. [0113]. Another exemplary assay is represented in FIG. 11, which includes LATE-PCR. An assay may include a pair of allele-specific primers each comprising a universal tail (ASP1 and ASP2), an excess of the shared locus-specific reverse primer (LSP), and a pair of universal FRET-based detector probes, one each for each of the two alleles....the presence and/or amount of the universal FRET-based detector probes are detected by detecting the fluorescence of "F" and/or "V", upon hybridization of the universal FRET-based detector probe(s) to the complementary tail of ASP1 and ASP2, respectively, which reduces the quenching effect of "Q").

Regarding Claim 37, Life Technologies discloses the method of claim 1, wherein the mixture further comprises a second nucleic acid primer (Para. [0007]. In certain embodiments, oligonucleotides are provided that are specific for a particular locus of the target nucleic acid. Such oligonucleotides are referred to herein as locus-specific primers or LSPs. In certain embodiments, the locus-specific primer may serve as a reverse primer in the amplification and genotyping methods disclosed herein).

Regarding Claim 38, Life Technologies discloses the method of claim 37, wherein said second nucleic acid primer is a target specific primer (Para. [0007]. In certain embodiments, oligonucleotides are provided that are specific for a particular locus of the target nucleic acid. Such oligonucleotides are referred to herein as locus-specific primers or LSPs. In certain embodiments, the locus-specific primer may serve as a reverse primer in the amplification and genotyping methods disclosed herein).

Regarding Claim 39, Life Technologies discloses the method of claim 37, wherein said second nucleic acid primer is configured to hybridize with said target nucleic acid or a derivative thereof and not to hybridize with said non-target nucleic or derivative thereof (Para. [0056]. The term "selectively hybridize" and variations thereof, means that, under appropriate stringency conditions, a given sequence (for example, but not limited to, a primer or probe) anneals with a second sequence comprising a complementary String of nucleotides (for example, but not limited to, a target flanking sequence or primer binding site of an amplicon), but does not anneal to undesired sequences, such as non-target nucleic acids, probes, or other primers).

Regarding Claim 40, Life Technologies discloses the method of claim 37, wherein the sequence of said target nucleic acid and the sequence of said non-target nucleic acid are different by at least one nucleotide (Para. [0006]. Provided herein are oligonucleotides, compositions, reaction mixtures, methods and kits for genotyping analysis, including, but not limited to SNP genotyping analysis; Para. [0083]. As used herein, the term "single nucleotide polymorphism" (SNP) refers to a DNA sequence variation occurring when a single nucleotide, e.g., A, T, C, or G, in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA contain a difference in a single nucleotide).

Regarding Claim 41, Life Technologies discloses the method of claim 37, wherein the sequence of said target nucleic acid and the sequence of said non-target nucleic acid are different by only one nucleotide (Para. [0006]. Provided herein are oligonucleotides, compositions, reaction mixtures, methods and kits for genotyping analysis, including, but not limited to SNP genotyping analysis; Para. [0083]. As used herein, the term "single nucleotide polymorphism" (SNP) refers to a DNA sequence variation occurring when a single nucleotide, e.g., A, T, C, or G, in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA contain a difference in a single nucleotide).

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Regarding Claim 42, Life Technologies discloses the method of claim 37, wherein the sequence of said target nucleic acid and the sequence of said non-target nucleic acid are different by no more than five nucleotides (Para. [0006], Provided herein are oligonucleotides, compositions, reaction mixtures, methods and kits for genotyping analysis, including, but not limited to SNP genotyping analysis; Para. [0083]. As used herein, the term "single nucleotide polymorphism" (SNP) refers to a DNA sequence variation occurring when a single nucleotide, e.g., A, T, C, or G, in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA contain a difference in a single nucleotide).

Regarding Claim 43, Life Technologies discloses the method of claim 38, wherein said target specific primer is homologous or complementary to a sequence of said target nucleic acid (Para. [0007]. Such oligonucleotides are referred to herein as allele-specific primers or ASPs. Such allele-specific primers include an allele-specific portion and a 5'-universal tail).

Regarding Claim 44, Life Technologies discloses the method of claim 37, wherein said second nucleic acid primer is a universal primer (Para. [0068]. According to certain embodiments, the amplification of nucleic acid sequences may involve the use of locus-specific, group-specific, and/or allele-specific primers. Locus-specific primers (LSPs) may be used to amplify all alleles encoded at a given locus (i.e. the primer is universal for all the alleles of a locus)).

Regarding Claim 45, Life Technologies discloses the method of claim 44, wherein said universal primer is complementary or homologous to a sequence of said second region (Para. [0007]. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer).

Regarding Claim 46, Life Technologies discloses the method of claim 37, wherein said second nucleic acid primer is configured to digest said cleavable, signal generating probe upon thermocycling (Para. [0012], the fluorescence may be further increased when the probe is digested by 5'-nuclease activity of polymerase; Para. [0115]. Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification); Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein).

Regarding Claim 47, Life Technologies discloses the method of claim 1, wherein said signal generating nucleic acid probe comprises a sequence homologous or complementary to said target nucleic acid (Para. [0032], FIGS. 7A through 7H: Performance comparison between certain of the UFP-based genotyping assays described herein and standard TaqMan(R) SNP assays (said standard assay comprising a labelled taqman probe complementary to an SNP target). Allele discrimination plots from universal reporter-based genotyping assays were compared to TaqMan(R) SNP assays; Para. [0115], exemplary methods for polymerizing and/or amplifying and detecting nucleic acids suitable for use as described herein are commercially available as TaqMan(R) assays ...an oligonucleotide probe capable of hybridizing to said target polynucleotide 3' relative to the primer. The oligonucleotide probe typically includes a detectable label (e.g., a fluorescent reporter molecule) and a quencher molecule capable of quenching the fluorescence of the reporter molecule).

Regarding Claim 49, Life Technologies discloses the method of claim 1, wherein said signal generating nucleic acid probe is configured to hybridize to said second region or said first region (Para. [0007]. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer).

Regarding Claim 50, Life Technologies discloses the method of claim 1, wherein said signal generating nucleic acid probe is configured to hybridize to only said first region (Para. [0032], FIGS. 7A through 7H: Performance comparison between certain of the UFP-based genotyping assays described herein and standard TaqMan(R) SNP assays (said standard assay comprising a labelled taqman probe complementary to an SNP target, i.e. only the first region). Allele discrimination plots from universal reporter-based genotyping assays were compared to TaqMan(R) SNP assays; Para. [0115], exemplary methods for polymerizing and/or amplifying and detecting nucleic acids suitable for use as described herein are commercially available as TaqMan(R) assays ...an oligonucleotide probe capable of hybridizing to said target polynucleotide 3' relative to the primer. The oligonucleotide probe typically includes a detectable label (e.g., a fluorescent reporter molecule) and a quencher molecule capable of quenching the fluorescence of the reporter molecule).

Regarding Claim 51, Life Technologies discloses the method of claim 1, wherein said signal generating nucleic acid probe is configured to hybridize to only said second region (Para. [0007]. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer).

Regarding Claim 52, Life Technologies discloses the method of claim 25, wherein said signal generating nucleic acid probe is configured to hybridize to said target-specific tail segment (Para. [0007]. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0106]. An exemplary SNP assay where at least one universal FRET-based detector probe replaces the UFP is shown in FIG. 9. An assay may include a pair of allele-specific primers where each allele specific includes a universal tail (ASP1 and ASP2), a shared locus-specific reverse primer (LSP), and a pair of universal detector probes for each of the two alleles (i.e. a unique probe for each allele)).

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Regarding Claim 53, Life Technologies discloses the method of claim 25, wherein said signal generating nucleic acid probe is configured to hybridize to said target-specific tail segment and not hybridize to a sequence, or complement thereof, of said target nucleic acid (Para. [0007]. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0106]. An exemplary SNP assay where at least one universal FRET-based detector probe replaces the UFP is shown in FIG. 9. An assay may include a pair of allele-specific primers where each allele specific includes a universal tail (ASP1 and ASP2), a shared locus-specific reverse primer (LSP), and a pair of universal detector probes for each of the two alleles (i.e. a unique probe for each allele)).

Regarding Claim 54, Life Technologies discloses the method of claim 25, wherein said target nucleic acid and said non-target a nucleic acid are different at a divergence location comprising at least one nucleotide, and said signal generating nucleic acid probe is configured to hybridize to said target specific tail segment and not hybridize to said divergence location comprising at least one nucleotide, or complement thereof (Para. [0006]. Provided herein are oligonucleotides, compositions, reaction mixtures, methods and kits for genotyping analysis, including, but not limited to SNP genotyping analysis; Para. [0007], the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1). In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0083]. As used herein, the term "single nucleotide polymorphism" (SNP) refers to a DNA sequence variation occurring when a single nucleotide, e.g., A, T, C, or G, in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA contain a difference in a single nucleotide).

Regarding Claim 55, Life Technologies discloses the method of claim 1, wherein said second region comprises: (A) a universal probe binding motif; and (B) a universal primer binding motif (Para. [0007], the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1). In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0012], upon hybridization of the universal FRET-based probe to its target sequence in the 5'-universal tail or the complement of the 5'-universal tail, the quenching of the fluorescence of the fluorophore is reduced).

Regarding Claim 56, Life Technologies discloses the method of claim 55, wherein said signal generating probe is configured to bind said universal probe binding motif (Para. [0007], the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1). In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0012], upon hybridization of the universal FRET-based probe to its target sequence in the 5'-universal tail or the complement of the 5'-universal tail, the quenching of the fluorescence of the fluorophore is reduced).

Regarding Claim 57, Life Technologies discloses the method of claim 55, wherein said mixture further comprise a universal primer configured to bind said universal primer binding motif (Para. [0068]. According to certain embodiments, the amplification of nucleic acid sequences may involve the use of locus-specific, group-specific, and/or allele-specific primers. Locus-specific primers (LSPs) may be used to amplify all alleles encoded at a given locus (i.e. the primer is universal for all the alleles of a locus)).

Regarding Claim 58, Life Technologies discloses the method of claim 55, wherein said target-specific segment is positioned on the 3' end of said forward primer (Para. [0007], oligonucleotides are provided that are specific for a particular allele or polymorphism. Such oligonucleotides are referred to herein as allele-specific primers or ASPs. Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1)).

Regarding Claim 59, Life Technologies discloses the method of claim 55, wherein said universal primer binding motif is located on the 5' end of said universal tail primer (Para. [0007], In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer).

Regarding Claim 60, Life Technologies discloses the method of claim 55, wherein said universal probe binding motif is flanked by the 5' end of said first region, and the 3' end of said universal primer binding motif (Para. [0007], Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1).)...the 3'-end of the allele-specific primer is extendable by a nucleic acid polymerase enzyme; Para. [0056]. The term "selectively hybridize" and variations thereof, means that, under appropriate stringency conditions, a given sequence (for example, but not limited to, a primer or probe) anneals with a second sequence comprising a complementary String of nucleotides (for example, but not limited to, a target flanking sequence or primer binding site of an amplicon), but does not anneal to undesired sequences, such as non-target nucleic acids, probes, or other primers).

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Regarding Claim 61, Life Technologies discloses the method of claim 55, wherein a plurality of forward primers are used, each universal primer binding motif comprises identical nucleotide sequences (Para. [0052]. In certain embodiments, an amplification reaction comprises multiplex amplification, in which a multiplicity of different target nucleic acids and/or a multiplicity of different amplification product species are simultaneously amplified using a multiplicity of different primer sets; Para. [0098], oligonucleotides are provided that are specific for a particular allele or polymorphism. Such oligonucleotides are referred to herein as allele-specific primers or ASPs. Such allele-specific primers comprise an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail comprises a binding site for a universal FRET-based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1). In certain embodiments, the 5'-universal tail comprises a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail comprises a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer).

Regarding Claim 62, Life Technologies discloses the method of claim 55, wherein a plurality of forward primers are used, each universal primer binding motifs comprises dissimilar nucleotide sequences (Para. [0013], a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP).

Regarding Claim 63, Life Technologies discloses the method of claim 55, wherein said second region comprises a second universal probe binding motif (Para. [0013], a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP).

Regarding Claim 64, Life Technologies discloses the method of claim 55, wherein said mixture further comprises a probe with a sequence complementary or homologous to said second universal probe binding motif (Para. [0007]. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0013], a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP).

Regarding Claim 65, Life Technologies discloses the method of claim 63, wherein said universal probe binding motif and said second universal probe binding motif comprise the same sequence (Para. [0007]. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0013], a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP).

Regarding Claim 66, Life Technologies discloses the method of claim 63, wherein said universal probe binding motif and said second universal probe binding motif comprise a different sequence (Para. [0007]. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0013], a first 5'-universal tail which includes a binding site for a first UFP... a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP).

Regarding Claim 67, Life Technologies discloses the method of claim 63, wherein said universal probe binding motif and said second universal probe binding motif are configured to bind the same probe sequence (Para. [0013], a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP).

Regarding Claim 68, Life Technologies discloses the method of claim 63, wherein said universal probe binding motif and said second universal probe binding motif are configured to bind a different probe sequence (Para. [0013], a first 5'-universal tail which includes a binding site for a first UFP... a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP).

Regarding Claim 70, Life Technologies discloses the method of claim 63, wherein a plurality of said forward primers are used, each universal primer binding motif comprises identical nucleotide sequences (Para. [0052]. In certain embodiments, an amplification reaction comprises multiplex amplification, in which a multiplicity of different target nucleic acids and/or a multiplicity of different amplification product species are simultaneously amplified using a multiplicity of different primer sets; Para. [0098], oligonucleotides are provided that are specific for a particular allele or polymorphism. Such oligonucleotides are referred to herein as allele-specific primers or ASPs. Such allele-specific primers comprise an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail comprises a binding site for a universal FRET-based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1). In certain embodiments, the 5'-universal tail comprises a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail comprises a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer).

Regarding Claim 71, Life Technologies discloses the method of claim 63, wherein said plurality of said forward primers are used, each universal primer binding motif comprises dissimilar nucleotide sequences (Para. [0013], a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP).

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Regarding Claim 72, Life Technologies discloses the method of claim 63, wherein said plurality of forward primers is used, each universal probe binding motif comprises a unique nucleotide sequence (Para. [0013], a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP; Para. [0052], a multiplicity of different amplification product species are simultaneously amplified using a multiplicity of different primer sets; Para. [0052], an amplification reaction comprises multiplex amplification, in which a multiplicity of different target nucleic acids and/or a multiplicity of different amplification product species are simultaneously amplified using a multiplicity of different primer sets; Para. [0069], each allele-specific primer may independently comprise a nucleotide sequence comprising a 5'-universal tail; Para. [0106], An exemplary SNP assay where at least one universal FRET-based detector probe replaces the UFP is shown in FIG. 9. An assay may include a pair of allele-specific primers where each allele specific includes a universal tail (ASP1 and ASP2), a shared locus-specific reverse primer (LSP), and a pair of universal detector probes for each of the two alleles (i.e. a unique probe for each allele)).

Regarding Claim 73, Life Technologies discloses a method for determining the presence or absence of a target nucleic acid in the presence of a non-target nucleic acid (Abstract, The present disclosure is drawn to methods for detection, quantitation and analysis of nucleotides of interest, for example SNPs, in nucleic acid sequences of interest using universal FRET-based reporter primers), said method comprising: (A) providing a sample comprising said target nucleic acid (Para. [0076], The target nucleic acid may be obtained from any Source, and may comprise any number of different compositional components.... target nucleic acids may be isolated from Samples using any of a variety of procedures known in the art); (B) forming a mixture comprising: i. said sample; ii. a non-extendible nucleic acid primer, wherein said non-extendible nucleic acid primer comprises a first region configured to hybridize to said target nucleic acid and configured not to bind to said non-target nucleic acid, and a second region configured to not hybridize to said target nucleic acid (Para. [0007], oligonucleotides are provided that are specific for a particular allele or polymorphism. Such oligonucleotides are referred to herein as allele-specific primers or ASPs. Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1). In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer...In certain embodiments, the 3' end of the allele-specific primer includes a blocking agent; Para. [0021], the composition and/or reaction mixture includes one or more target nucleic acid molecules and a first allele-specific primer, which is specific for a first allele, including a first 5'-universal tail which includes a binding site for a first UFP), and wherein said non-extendible nucleic acid primer comprises a target-specific RNA base, DNA base, or unnatural base, configured to, when said first region is hybridized to said target nucleic acid, form a base hybrid pair at a target base of said target nucleic acid and not form said base hybrid pair at a corresponding base in the non-target nucleic acid (Para. [0007], In certain embodiments, the 3' end of the allele-specific primer includes a blocking agent such that the blocked end may be activated by a polyphosphorolysis enzyme. In certain embodiments, the blocking agent is a dideoxynucleotide (ddN)); and iii. a signal generating nucleic acid probe (Para. [0012], oligonucleotides are provided that may be used to detect the amplification of a target nucleic acid and/or may be used to monitor or detect the amplification of an allele or polymorphism in a target nucleic acid. Such oligonucleotides are referred to herein as "universal FRET-based detector probes" the fluorescence may be further increased when the probe is digested by 5'-nuclease activity of polymerase; Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein); (C) subjecting said mixture to cleavage conditions such that nucleic acids comprising said base hybrid pair are cleaved such that said non-extendible nucleic acid is cleaved, thereby converting said non-extendible nucleic acid primer to an extendible primer (Para. [0007], In certain embodiments, the 3' end of the allele-specific primer includes a blocking agent such that the blocked end may be activated by a polyphosphorolysis enzyme); (D) subjecting said mixture to said amplification conditions, said amplification conditions appropriate to amplify said target nucleic acid with an amplification reaction, such that said signal generating nucleic acid probe is degraded and a signal is generated if said target nucleic acid is present in said mixture (Para. [0115], TaqMan R assays are typically carried out by performing nucleic acid amplification on a target polynucleotide using a nucleic acid polymerase having 5'-to-3' nuclease activity, a primer capable of hybridizing to the target polynucleotide, and an oligonucleotide probe.... Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification)); and (E) detecting the presence or absence of said signal, thereby determining the presence or absence of said target nucleic acid in the presence of said non-target nucleic acid (Para. [0115], The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification)).

Regarding Claim 74, Life Technologies discloses the method of claim 73, wherein said amplification reaction comprises a reverse transcriptase polymerase chain reaction (RT-PCR) or a polymerase chain reaction (PCR) (Para. [0046], The amplification reaction may be a polymerase-mediated extension reaction Such as, for example, a polymerase chain reaction (PCR)).

Regarding Claim 75, Life Technologies discloses the method of claim 73, wherein said target-specific RNA base, DNA base, or unnatural base of said non-extendible nucleic acid primer is at a location complementary to said target nucleic acid (Para. [0007], In certain embodiments, the 3' end of the allele-specific primer includes a blocking agent such that the blocked end may be activated by a polyphosphorolysis enzyme. In certain embodiments, the blocking agent is a dideoxynucleotide (ddN)).

Regarding Claim 77, Life Technologies discloses the method of claim 73, wherein said base hybrid pair is a DNA:RNA pair, a DNA:unnatural base, or a RNA:unnatural base pair (Para. [0007], In certain embodiments, the 3' end of the allele-specific primer includes a blocking agent such that the blocked end may be activated by a polyphosphorolysis enzyme. In certain embodiments, the blocking agent is a dideoxynucleotide (ddN)).

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Regarding Claim 78, Life Technologies discloses the method of claim 73, wherein in c), said non-extendible nucleic acid primer is cleaved at a location adjacent to or at said base hybrid pair (Para. [0094], Family I DNA polymerases may be used according to certain embodiments of the methods disclosed herein when the activatable oligonucleotide comprises a dideoxynucleotide at the 3'-end or an acyclonucleotide at the 3'-end. When the activatable oligonucleotide is an acyclonucleotide, Family II archaeon DNA polymerases may be used.... Family I DNA polymerases may be used according to certain embodiments of the methods disclosed herein when the activatable oligonucleotide comprises a dideoxynucleotide at the 3'-end or an acyclonucleotide at the 3'-end. When the activatable oligonucleotide is an acyclonucleotide, Family II archaeon DNA polymerases may be used).

Regarding Claim 79, Life Technologies discloses the method of claim 73, wherein said cleavage conditions comprise an additional reagent or enzyme for cleaving said first non-extendible nucleic acid primer (Para. [0094], any enzyme that can perform pyrophosphorolysis-activated polymerization reactions ...the DNA polymerase used may also have pyrophosphorolysis activity.... In certain embodiments, two or more nucleic acid polymerases may also be used in one reaction).

Regarding Claim 81, Life Technologies discloses the method of claim 73, wherein said target nucleic acid is an allele (Para. [0007], oligonucleotides are provided that are specific for a particular allele or polymorphism. Such oligonucleotides are referred to herein as allele-specific primers or ASPs. Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer).

Regarding Claim 82, Life Technologies discloses the method of claim 73, wherein said target nucleic acid is a mutant sequence and said non-target nucleic acid is a wild type sequence (Para. [0151], The methods described herein may be also be used to detect rare alleles in the presence of wild type alleles (e.g., one mutant allele in the presence of 10⁹-10¹⁰ wild type alleles)).

Regarding Claim 83, Life Technologies discloses the method of claim 73, wherein the sequence of said target nucleic acid and the sequence of said non-target nucleic acid is different at least one nucleotide (Para. [0006], Provided herein are oligonucleotides, compositions, reaction mixtures, methods and kits for genotyping analysis, including, but not limited to SNP genotyping analysis; Para. [0083], As used herein, the term "single nucleotide polymorphism" (SNP) refers to a DNA sequence variation occurring when a single nucleotide, e.g., A, T, C, or G, in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA contain a difference in a single nucleotide).

Regarding Claim 84, Life Technologies discloses the method of claim 73, wherein the sequence of said target nucleic acid and the sequence of said non-target nucleic acid is different by only one nucleotide (Para. [0006], Provided herein are oligonucleotides, compositions, reaction mixtures, methods and kits for genotyping analysis, including, but not limited to SNP genotyping analysis; Para. [0083], As used herein, the term "single nucleotide polymorphism" (SNP) refers to a DNA sequence variation occurring when a single nucleotide, e.g., A, T, C, or G, in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA contain a difference in a single nucleotide).

Regarding Claim 85, Life Technologies discloses the method of claim 73, wherein the sequence of said target nucleic acid and the sequence of said non target nucleic acid is different by no more than five nucleotides (Para. [0006], Provided herein are oligonucleotides, compositions, reaction mixtures, methods and kits for genotyping analysis, including, but not limited to SNP genotyping analysis; Para. [0083], As used herein, the term "single nucleotide polymorphism" (SNP) refers to a DNA sequence variation occurring when a single nucleotide, e.g., A, T, C, or G, in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA contain a difference in a single nucleotide).

Regarding Claim 86, Life Technologies discloses the method of claim 73, wherein said extendible primer is specific to and selectively amplifies only said target nucleic acid (Para. [0056], The term "selectively hybridize" and variations thereof, means that, under appropriate stringency conditions, a given sequence (for example, but not limited to, a primer or probe) anneals with a second sequence comprising a complementary String of nucleotides (for example, but not limited to, a target flanking sequence or primer binding site of an amplicon), but does not anneal to undesired sequences, such as non-target nucleic acids, probes, or other primers; Para. [0130], the desired nucleic acid strand of the target allele-specific DNA synthesized on the template strand is amplified preferentially over any nucleic acid strand synthesized on the non-target allele-specific DNA).

Regarding Claim 87, Life Technologies discloses the method of claim 73, wherein said mixture does not employ the use of a nucleic acid or peptide blocking agents (Para. [0007], In certain embodiments (only in certain embodiments), the 3' end of the allele-specific primer includes a blocking agent Such that the blocked end may be activated by a pyro phosphorolysis enzyme. In certain embodiments, the 3'-end of the allele-specific primer is extendable(not blocked) by a nucleic acid polymerase enzyme).

Regarding Claim 88, Life Technologies discloses the method of claim 73, wherein said mixture further comprising a reverse primer (Para. [0007], In certain embodiments, oligonucleotides are provided that are specific for a particular locus of the target nucleic acid. Such oligonucleotides are referred to herein as locus-specific primers or LSPs. In certain embodiments, the locus-specific primer may serve as a reverse primer in the amplification and genotyping methods disclosed herein).

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Regarding Claim 89, Life Technologies discloses the method of claim 73, wherein said extendible primer is configured to hybridize to a first area of a nucleic acid sequence of said target nucleic acid, and said reverse primer is configured to hybridize to a second area of the nucleic acid sequence of said target nucleic acid, thereby being configured to amplify the nucleic acid sequence under conditions sufficient for nucleic acid amplification (Para. [0007]. In certain embodiments, the 3'-end of the allele-specific primer is extendable by a nucleic acid polymerase enzyme....In certain embodiments, oligonucleotides are provided that are specific for a particular locus of the target nucleic acid. Such oligonucleotides are referred to herein as locus-specific primers or LSPs. In certain embodiments, the locus-specific primer may serve as a reverse primer in the amplification and genotyping methods disclosed herein).

Regarding Claim 90, Life Technologies discloses the method of claim 89, wherein said first area comprises a 3' end of said nucleic acid sequence (Fig. 1, depicting the allele specific primer extending from the 3' end of the target and, and the locus specific or reverse primer extending from the opposite end (i.e. 5' end) of the target).

Regarding Claim 91, Life Technologies discloses the method of claim 89, wherein said second area comprises a 5' end of said nucleic acid sequence (Fig. 1, depicting the allele specific primer extending from the 3' end of the target and, and the locus specific or reverse primer extending from the opposite end (i.e. 5' end) of the target).

Regarding Claim 92, Life Technologies discloses the method of claim 88, wherein said reverse primer comprises a sequence with complementarity or is homologous to a sequence on both target nucleic acid and nontarget nucleic acid (Para. [0068]. According to certain embodiments, the amplification of nucleic acid sequences may involve the use of locus-specific, group-specific, and/or allele-specific primers. Locus-specific primers (LSPs) may be used to amplify all alleles encoded at a given locus).

Regarding Claim 93, Life Technologies discloses the method of claim 88, wherein said reverse primer is a locus specific primer (Para. [0007]. In certain embodiments, the 3'-end of the allele-specific primer is extendable by a nucleic acid polymerase enzyme....In certain embodiments, oligonucleotides are provided that are specific for a particular locus of the target nucleic acid. Such oligonucleotides are referred to herein as locus-specific primers or LSPs. In certain embodiments, the locus-specific primer may serve as a reverse primer in the amplification and genotyping methods disclosed herein).

Regarding Claim 94, Life Technologies discloses the method of claim 88, wherein said reverse primer is a universal primer (Para. [0068]. According to certain embodiments, the amplification of nucleic acid sequences may involve the use of locus-specific, group-specific, and/or allele-specific primers. Locus-specific primers (LSPs) may be used to amplify all alleles encoded at a given locus (i.e. the primer is universal for all the alleles of a locus)).

Regarding Claim 95, Life Technologies discloses the method of claim 88, wherein said reverse primer is configured such that upon said thermally cycling said signal generating nucleic acid probe is digested (Para. [0012], oligonucleotides are provided that may be used to detect the amplification of a target nucleic acid and/or may be used to monitor or detect the amplification of an allele or polymorphism in a target nucleic acid. Such oligonucleotides are referred to herein as "universal FRET-based detector probes" the fluorescence may be further increased when the probe is digested by 5'-nuclease activity of polymerase; Para. [0115]. Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification); Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein).

Regarding Claim 96, Life Technologies discloses the method of claim 73, wherein said mixture further comprises a nucleic acid enzyme (Para. [0012], oligonucleotides are provided that may be used to detect the amplification of a target nucleic acid and/or may be used to monitor or detect the amplification of an allele or polymorphism in a target nucleic acid. Such oligonucleotides are referred to herein as "universal FRET-based detector probes" the fluorescence may be further increased when the probe is digested by 5'-nuclease activity of polymerase; Para. [0115]. Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification); Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein).

Regarding Claim 97, Life Technologies discloses the method of claim 73, wherein said amplification reaction is a polymerase chain reaction (PCR) (Para. [0012], oligonucleotides are provided that may be used to detect the amplification of a target nucleic acid and/or may be used to monitor or detect the amplification of an allele or polymorphism in a target nucleic acid. Such oligonucleotides are referred to herein as "universal FRET-based detector probes" the fluorescence may be further increased when the probe is digested by 5'-nuclease activity of polymerase; Para. [0115]. Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification); Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein).

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Regarding Claim 98, Life Technologies discloses the method of claim 97, wherein said PCR is a quantitative polymerase chain reaction (qPCR) (Para. [0012], oligonucleotides are provided that may be used to detect the amplification of a target nucleic acid and/or may be used to monitor or detect the amplification of an allele or polymorphism in a target nucleic acid. Such oligonucleotides are referred to herein as "universal FRET-based detector probes" the fluorescence may be further increased when the probe is digested by 5'-nuclease activity of polymerase; Para. [0115], Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification); Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein).

Regarding Claim 99, Life Technologies discloses the method of claim 73, wherein said amplification conditions comprises: a dNTP, a salt, a buffer, or a combination thereof (Para. [0159], In some embodiments, multiple primer sets are included. In one embodiment, the kit may include one or more of, for example, a buffer (e.g., Tris), one or more salts (e.g., KCl), glycerol, dNTPs (dA, dT, dG, dC, du), recombinant BSA (bovine serum albumin)).

Regarding Claim 100, Life Technologies discloses the method of claim 73, wherein said signal generating nucleic acid probe further comprises a signal tag (Para. [0084]; a reaction vessel comprises a well of a multi-well plate, a spot on a glass slide, or a channel or chamber of a microfluidics device, including without limitation a TaqMan(R) Low Density Array or a TaqMan(R) Open Array Real-Time PCR plate; Para. [0115], Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification); Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein).

Regarding Claim 101, Life Technologies discloses the method of claim 100, wherein said signal tag generates said signal (Para. [0084]; a reaction vessel comprises a well of a multi-well plate, a spot on a glass slide, or a channel or chamber of a microfluidics device, including without limitation a TaqMan(R) Low Density Array or a TaqMan(R) Open Array Real-Time PCR plate; Para. [0115], Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification); Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein).

Regarding Claim 102, Life Technologies discloses the method of claim 101, wherein said signal tag generates said signal upon degradation of said signal generating nucleic acid probe by the 5' to 3' exonuclease activity of said nucleic acid enzyme, thereby liberating said signal for detection by a real time PCR instrument (Para. [0084]; a reaction vessel comprises a well of a multi-well plate, a spot on a glass slide, or a channel or chamber of a microfluidics device, including without limitation a TaqMan(R) Low Density Array or a TaqMan(R) Open Array Real-Time PCR plate; Para. [0115], Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification); Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein).

Regarding Claim 104, Life Technologies discloses the method of claim 73, wherein said second region comprises: (A) a target-specific tail segment; and (B) a universal tail segment (Para. [0007], Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP) and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1)).

Regarding Claim 105, Life Technologies discloses the method of claim 73, wherein said first region is positioned on the 3' end of extendible primer (Para. [0007], Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP) and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1)....the 3'-end of the allele-specific primer is extendable by a nucleic acid polymerase enzyme).

Regarding Claim 106, Life Technologies discloses the method of claim 104, wherein said universal tail segment is located on the 5' end of said extendible primer (Para. [0007], Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP) and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1)....the 3'-end of the allele-specific primer is extendable by a nucleic acid polymerase enzyme).

Regarding Claim 107, Life Technologies discloses the method of claim 104, wherein said target-specific tail segment is flanked on the 3' by said target-specific segment, and flanked on the 5' end by said universal tail segment on said non-extendible primer (Para. [0007], Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP) and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1)....the 3'-end of the allele-specific primer is extendable by a nucleic acid polymerase enzyme; Para. [0056], The term "selectively hybridize" and variations thereof, means that, under appropriate stringency conditions, a given sequence (for example, but not limited to, a primer or probe) anneals with a second sequence comprising a complementary String of nucleotides (for example, but not limited to, a target flanking sequence or primer binding site of an amplicon), but does not anneal to undesired sequences, such as non-target nucleic acids, probes, or other primers).

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Regarding Claim 108, Life Technologies discloses the method of claim 104, wherein a plurality of non-extendible primers is used, wherein each second region of said non-extendible primers comprises identical nucleotide sequences (Para. [0052], In certain embodiments, an amplification reaction comprises multiplex amplification, in which a multiplicity of different target nucleic acids and/or a multiplicity of different amplification product species are simultaneously amplified using a multiplicity of different primer sets; Para. [0098], oligonucleotides are provided that are specific for a particular allele or polymorphism. Such oligonucleotides are referred to herein as allele-specific primers or ASPs. Such allele-specific primers comprise an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail comprises a binding site for a universal FRET-based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1). In certain embodiments, the 5'-universal tail comprises a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail comprises a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer).

Regarding Claim 109, Life Technologies discloses the method of claim 104, wherein a plurality of non-extendible primers is used, wherein each second region of said non-extendible primers comprises dissimilar nucleotide sequences (Para. [0013], a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP).

Regarding Claim 110, Life Technologies discloses the method of claim 104, wherein a plurality of non-extendible primers is used, wherein each second region of said non-extendible primers comprises unique nucleotide sequences (Para. [0013], a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP; Para. [0052], a multiplicity of different amplification product species are simultaneously amplified using a multiplicity of different primer sets; Para. [0052], an amplification reaction comprises multiplex amplification, in which a multiplicity of different target nucleic acids and/or a multiplicity of different amplification product species are simultaneously amplified using a multiplicity of different primer sets; Para. [0069], each allele-specific primer may independently comprise a nucleotide sequence comprising a 5'-universal tail; Para. [0106], An exemplary SNP assay where at least one universal FRET-based detector probe replaces the UFP is shown in FIG. 9. An assay may include a pair of allele-specific primers where each allele specific includes a universal tail (ASP1 and ASP2), a shared locus-specific reverse primer (LSP), and a pair of universal detector probes for each of the two alleles (i.e. a unique probe for each allele)).

Regarding Claim 111, Life Technologies discloses the method of claim 73, wherein said signal generating nucleic acid probe comprises sequence complementary or homologous to said non-extendible primer (Para. [0007], In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer).

Regarding Claim 112, Life Technologies discloses the method of claim 73, wherein said signal generating nucleic acid probe is a target-specific probe (Para. [0106], An exemplary SNP assay where at least one universal FRET-based detector probe replaces the UFP is shown in FIG. 9. An assay may include a pair of allele-specific primers where each allele specific includes a universal tail (ASP1 and ASP2), a shared locus-specific reverse primer (LSP), and a pair of universal detector probes for each of the two alleles (i.e. a unique probe for each allele)).

Regarding Claim 115, Life Technologies discloses the method of claim 73, wherein said probe binds to said target nucleic acid in an amplicon generated by second strand synthesis initiated in said amplification reaction (Para. [0113], Another exemplary assay is represented in FIG. 11, which includes LATE-PCR. An assay may include a pair of allele-specific primers each comprising a universal tail (ASP1 and ASP2), an excess of the shared locus-specific reverse primer (LSP), and a pair of universal FRET-based detector probes, one each for each of the two alleles....the presence and/or amount of the universal FRET-based detector probes are detected by detecting the fluorescence of "F" and/or "V", upon hybridization of the universal FRET-based detector probe(s) to the complementary tail of ASP1 and ASP2, respectively, which reduces the quenching effect of "Q").

Regarding Claim 116, Life Technologies discloses the method of claim 73, wherein the mixture further comprises a second nucleic acid primer (Para. [0007], In certain embodiments, oligonucleotides are provided that are specific for a particular locus of the target nucleic acid. Such oligonucleotides are referred to herein as locus-specific primers or LSPs. In certain embodiments, the locus-specific primer may serve as a reverse primer in the amplification and genotyping methods disclosed herein).

Regarding Claim 117, Life Technologies discloses the method of claim 116, wherein said second nucleic acid primer is a target specific primer (Para. [0007], In certain embodiments, oligonucleotides are provided that are specific for a particular locus of the target nucleic acid. Such oligonucleotides are referred to herein as locus-specific primers or LSPs. In certain embodiments, the locus-specific primer may serve as a reverse primer in the amplification and genotyping methods disclosed herein).

Regarding Claim 118, Life Technologies discloses the method of claim 116, wherein said second nucleic acid primer is configured to hybridize with said target nucleic acid or a derivative thereof and not to hybridize with said non-target nucleic acid or derivative thereof (Para. [0056], The term "selectively hybridize" and variations thereof, means that, under appropriate stringency conditions, a given sequence (for example, but not limited to, a primer or probe) anneals with a second sequence comprising a complementary String of nucleotides (for example, but not limited to, a target flanking sequence or primer binding site of an amplicon), but does not anneal to undesired sequences, such as non-target nucleic acids, probes, or other primers).

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Regarding Claim 119, Life Technologies discloses the method of claim 116, wherein the sequence of said target nucleic acid and the sequence of said non-target nucleic acid are different by at least one nucleotide (Para. [0006], Provided herein are oligonucleotides, compositions, reaction mixtures, methods and kits for genotyping analysis, including, but not limited to SNP genotyping analysis; Para. [0083], As used herein, the term "single nucleotide polymorphism" (SNP) refers to a DNA sequence variation occurring when a single nucleotide, e.g., A, T, C, or G, in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA contain a difference in a single nucleotide).

Regarding Claim 120, Life Technologies discloses the method of claim 116, wherein the sequence of said target nucleic acid and the sequence of said non-target nucleic acid are different by only one nucleotide (Para. [0006], Provided herein are oligonucleotides, compositions, reaction mixtures, methods and kits for genotyping analysis, including, but not limited to SNP genotyping analysis; Para. [0083], As used herein, the term "single nucleotide polymorphism" (SNP) refers to a DNA sequence variation occurring when a single nucleotide, e.g., A, T, C, or G, in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA contain a difference in a single nucleotide).

Regarding Claim 121, Life Technologies discloses the method of claim 116, wherein the sequence of said target nucleic acid and the sequence of said non-target nucleic acid are different by no more than five nucleotides (Para. [0006], Provided herein are oligonucleotides, compositions, reaction mixtures, methods and kits for genotyping analysis, including, but not limited to SNP genotyping analysis; Para. [0083], As used herein, the term "single nucleotide polymorphism" (SNP) refers to a DNA sequence variation occurring when a single nucleotide, e.g., A, T, C, or G, in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA contain a difference in a single nucleotide).

Regarding Claim 122, Life Technologies discloses the method of claim 117, wherein said target specific primer is homologous or complementary to a sequence of said target nucleic acid (Para. [0007], Such oligonucleotides are referred to herein as allele-specific primers or ASPs. Such allele-specific primers include an allele-specific portion and a 5'-universal tail).

Regarding Claim 123, Life Technologies discloses the method of claim 116, wherein said second nucleic acid primer is a universal primer (Para. [0068], According to certain embodiments, the amplification of nucleic acid sequences may involve the use of locus-specific, group-specific, and/or allele-specific primers. Locus-specific primers (LSPs) may be used to amplify all alleles encoded at a given locus (i.e. the primer is universal for all the alleles of a locus)).

Regarding Claim 124, Life Technologies discloses the method of claim 123, wherein said universal primer is complementary or homologous to a sequence of said second region (Para. [0007], In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer).

Regarding Claim 125, Life Technologies discloses the method of claim 116, wherein said second nucleic acid primer is configured to digest said cleavable, signal generating probe upon thermocycling (Para. [0012], the fluorescence may be further increased when the probe is digested by 5'-nuclease activity of polymerase; Para. [0115], Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification); Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein).

Regarding Claim 126, Life Technologies discloses the method of claim 73, wherein said signal generating probe comprises a sequence homologous or complementary to said target nucleic acid (Para. [0032], FIGS. 7A through 7H: Performance comparison between certain of the UFP-based genotyping assays described herein and standard TaqMan(R) SNP assays (said standard assay comprising a labelled taqman probe complementary to an SNP target). Allele discrimination plots from universal reporter-based genotyping assays were compared to TaqMan(R) SNP assays; Para. [0115], exemplary methods for polymerizing and/or amplifying and detecting nucleic acids suitable for use as described herein are commercially available as TaqMan(R) assays ...an oligonucleotide probe capable of hybridizing to said target polynucleotide 3' relative to the primer. The oligonucleotide probe typically includes a detectable label (e.g., a fluorescent reporter molecule) and a quencher molecule capable of quenching the fluorescence of the reporter molecule).

Regarding Claim 128, Life Technologies discloses the method of claim 73, wherein said signal generating nucleic acid probe is configured to hybridize to said second region or said first region (Para. [0007], In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer).

Regarding Claim 129, Life Technologies discloses the method of claim 73, wherein said signal generating nucleic acid probe is configured to hybridize to only said first region (Para. [0032], FIGS. 7A through 7H: Performance comparison between certain of the UFP-based genotyping assays described herein and standard TaqMan(R) SNP assays (said standard assay comprising a labelled taqman probe complementary to an SNP target, i.e. only the first region). Allele discrimination plots from universal reporter-based genotyping assays were compared to TaqMan(R) SNP assays; Para. [0115], exemplary methods for polymerizing and/or amplifying and detecting nucleic acids suitable for use as described herein are commercially available as TaqMan(R) assays ...an oligonucleotide probe capable of hybridizing to said target polynucleotide 3' relative to the primer. The oligonucleotide probe typically includes a detectable label (e.g., a fluorescent reporter molecule) and a quencher molecule capable of quenching the fluorescence of the reporter molecule).

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Regarding Claim 130, Life Technologies discloses the method of claim 73, wherein said signal generating nucleic acid probe is configured to hybridize to only said second region (Para. [0007]. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer).

Regarding Claim 131, Life Technologies discloses the method of claim 104, wherein said signal generating nucleic acid probe is configured to hybridize to said target-specific tail segment (Para. [0007]. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0106]. An exemplary SNP assay where at least one universal FRET-based detector probe replaces the UFP is shown in FIG. 9. An assay may include a pair of allele-specific primers where each allele specific includes a universal tail (ASP1 and ASP2), a shared locus-specific reverse primer (LSP), and a pair of universal detector probes for each of the two alleles (i.e. a unique probe for each allele)).

Regarding Claim 132, Life Technologies discloses the method of claim 104, wherein said signal generating nucleic acid probe is configured to hybridize to said target-specific tail segment and not hybridize to a sequence, or complement thereof, of said target nucleic acid (Para. [0007]. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0106]. An exemplary SNP assay where at least one universal FRET-based detector probe replaces the UFP is shown in FIG. 9. An assay may include a pair of allele-specific primers where each allele specific includes a universal tail (ASP1 and ASP2), a shared locus-specific reverse primer (LSP), and a pair of universal detector probes for each of the two alleles (i.e. a unique probe for each allele)).

Regarding Claim 133, Life Technologies discloses the method of claim 104, wherein said target nucleic acid and said non-target a nucleic acid are different at a divergence location comprising at least one nucleotide, and said signal generating nucleic acid probe is configured to hybridize to said target-specific tail segment and not hybridize to said divergence location comprising at least one nucleotide, or complement thereof (Para. [0006]. Provided herein are oligonucleotides, compositions, reaction mixtures, methods and kits for genotyping analysis, including, but not limited to SNP genotyping analysis; Para. [0007], the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP) and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1). In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0083]. As used herein, the term "single nucleotide polymorphism" (SNP) refers to a DNA sequence variation occurring when a single nucleotide, e.g., A, T, C, or G, in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGCTTA to AAGCTTA contain a difference in a single nucleotide).

Regarding Claim 134, Life Technologies discloses the method of claim 73, wherein said second region comprises: (A) a universal probe binding motif, and (B) a universal primer binding motif (Para. [0007], the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP) and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1). In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0012], upon hybridization of the universal FRET-based probe to its target sequence in the 5'-universal tail or the complement of the 5'-universal tail, the quenching of the fluorescence of the fluorophore is reduced).

Regarding Claim 135, Life Technologies discloses the method of claim 134, wherein said signal generating probe is configured to bind said universal probe binding motif (Para. [0007], the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP) and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1). In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0012], upon hybridization of the universal FRET-based probe to its target sequence in the 5'-universal tail or the complement of the 5'-universal tail, the quenching of the fluorescence of the fluorophore is reduced).

Regarding Claim 136, Life Technologies discloses the method of claim 134, wherein said mixture further comprise a universal primer configured to bind said universal primer binding motif (Para. [0068]. According to certain embodiments, the amplification of nucleic acid sequences may involve the use of locus-specific, group-specific, and/or allele-specific primers. Locus-specific primers (LSPs) may be used to amplify all alleles encoded at a given locus (i.e. the primer is universal for all the alleles of a locus)).

Regarding Claim 137, Life Technologies discloses the method of claim 134, wherein said target-specific segment is positioned on the 3' end of said non-extendible primer (Para. [0007]. Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP) In certain embodiments, the 3' end of the allele-specific primer includes a blocking agent Such that the blocked end may be activated by a pyrophosphorolysis enzyme).

Regarding Claim 138, Life Technologies discloses the method of claim 134, wherein said universal primer binding motif is located on the 5' end of said universal tail primer (Para. [0007]. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer).

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Regarding Claim 139, Life Technologies discloses the method of claim 134, wherein said universal probe binding motif is flanked by the 5' end of said first region, and the 3' end of said universal primer binding motif (Para. [0007], Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1).)....the 3'-end of the allele-specific primer is extendable by a nucleic acid polymerase enzyme; Para. [0056], The term "selectively hybridize" and variations thereof, means that, under appropriate stringency conditions, a given sequence (for example, but not limited to, a primer or probe) anneals with a second sequence comprising a complementary String of nucleotides (for example, but not limited to, a target flanking sequence or primer binding site of an amplicon), but does not anneal to undesired sequences, such as non-target nucleic acids, probes, or other primers).

Regarding Claim 140, Life Technologies discloses the method of claim 134, wherein a plurality of non-extendible primers is used, each universal primer binding motif comprises identical nucleotide sequences (Para. [0052], In certain embodiments, an amplification reaction comprises multiplex amplification, in which a multiplicity of different target nucleic acids and/or a multiplicity of different amplification product species are simultaneously amplified using a multiplicity of different primer sets; Para. [0098], oligonucleotides are provided that are specific for a particular allele or polymorphism. Such oligonucleotides are referred to herein as allele-specific primers or ASPs. Such allele-specific primers comprise an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail comprises a binding site for a universal FRET-based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1). In certain embodiments, the 5'-universal tail comprises a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail comprises a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer).

Regarding Claim 141, Life Technologies discloses the method of claim 134, wherein a plurality of non-extendible primers is used, each universal primer binding motifs comprises dissimilar nucleotide sequences (Para. [0013], a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP).

Regarding Claim 142, Life Technologies discloses the method of claim 134, wherein said second region comprises a second universal probe binding motif (Para. [0013], a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP).

Regarding Claim 143, Life Technologies discloses the method of claim 134, wherein said mixture further comprises a probe with a sequence complementary or homologous to said second universal probe binding motif (Para. [0007], In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0013], a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP).

Regarding Claim 144, Life Technologies discloses the method of claim 143, wherein said universal probe binding motif and said second universal probe binding motif comprise the same sequence (Para. [0007], In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0013], a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP).

Regarding Claim 145, Life Technologies discloses the method of claim 143, wherein said universal probe binding motif and said second universal probe binding motif comprise a different sequence (Para. [0007], In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0013], a first 5'-universal tail which includes a binding site for a first UFP... a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP).

Regarding Claim 146, Life Technologies discloses the method of claim 143, wherein said universal probe binding motif and said second universal probe binding motif are configured to bind the same probe sequence (Para. [0013], a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP).

Regarding Claim 147, Life Technologies discloses the method of claim 143, wherein said universal probe binding motif and said second universal probe binding motif are configured to bind a different probe sequence (Para. [0013], a first 5'-universal tail which includes a binding site for a first UFP... a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP).

Regarding Claim 149, Life Technologies discloses the method of claim 143, wherein a plurality of said non-extendible primers are used, each universal primer binding motif comprises identical nucleotide sequences (Para. [0052], In certain embodiments, an amplification reaction comprises multiplex amplification, in which a multiplicity of different target nucleic acids and/or a multiplicity of different amplification product species are simultaneously amplified using a multiplicity of different primer sets; Para. [0098], oligonucleotides are provided that are specific for a particular allele or polymorphism. Such oligonucleotides are referred to herein as allele-specific primers or ASPs. Such allele-specific primers comprise an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail comprises a binding site for a universal FRET-based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1). In certain embodiments, the 5'-universal tail comprises a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail comprises a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer).

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Regarding Claim 150, Life Technologies discloses the method of claim 143, wherein said plurality of said non-extendible primers are used, each universal primer binding motifs comprises dissimilar nucleotide sequences (Para. [0013], a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP; Para. [0052], a multiplicity of different amplification product species are simultaneously amplified using a multiplicity of different primer sets; Para. [0052], an amplification reaction comprises multiplex amplification, in which a multiplicity of different target nucleic acids and/or a multiplicity of different amplification product species are simultaneously amplified using a multiplicity of different primer sets; Para. [0069], each allele-specific primer may independently comprise a nucleotide sequence comprising a 5'-universal tail; Para. [0106], An exemplary SNP assay where at least one universal FRET-based detector probe replaces the UFP is shown in FIG. 9. An assay may include a pair of allele-specific primers where each allele specific includes a universal tail (ASP1 and ASP2), a shared locus-specific reverse primer (LSP), and a pair of universal detector probes for each of the two alleles (i.e. a unique probe for each allele)).

Regarding Claim 151, Life Technologies discloses the method of claim 143, wherein said plurality of non-extendible primers is used, each of said at least two universal probe binding motifs comprises a unique nucleotide sequence (Para. [0013], a second allele-specific primer which is specific for a second allele, including a second 5'-universal tail which includes a binding site for a second UFP; Para. [0052], a multiplicity of different amplification product species are simultaneously amplified using a multiplicity of different primer sets; Para. [0052], an amplification reaction comprises multiplex amplification, in which a multiplicity of different target nucleic acids and/or a multiplicity of different amplification product species are simultaneously amplified using a multiplicity of different primer sets; Para. [0069], each allele-specific primer may independently comprise a nucleotide sequence comprising a 5'-universal tail; Para. [0106], An exemplary SNP assay where at least one universal FRET-based detector probe replaces the UFP is shown in FIG. 9. An assay may include a pair of allele-specific primers where each allele specific includes a universal tail (ASP1 and ASP2), a shared locus-specific reverse primer (LSP), and a pair of universal detector probes for each of the two alleles (i.e. a unique probe for each allele)).

Regarding Claim 152, Life Technologies discloses a kit for use in determining the presence or absence of a target nucleic acid in the presence of a non-target nucleic acid (Abstract, The present disclosure is drawn to methods for detection, quantitation and analysis of nucleotides of interest, for example SNPs, in nucleic acid sequences of interest using universal FRET-based reporter primers; Para. [0023], kits are provided that may be used to carry out hybridization, extension and amplification reactions using the oligonucleotides provided herein), said kit comprising: (A) a forward primer comprising a first region configured to hybridize to said target nucleic acid under amplification conditions and configured to not hybridize to said non-target nucleic acid under said amplification conditions, and a second region configured to not hybridize to said target nucleic acid under said amplification conditions; (B) a signal generating nucleic acid probe, wherein said signal generating nucleic acid probe anneals to said second region, or regions complementary thereto, when subjected to said amplification conditions (Para. [0023], The kits described herein may include one or more components selected from the group consisting of one or more oligonucleotides described herein, including but not limited to, one or more allele-specific primer, one or more universal FRET based reporter primer, and one or more locus specific primer; one or more nucleic acid polymerase, Such as a thermostable polymerase.... a first allele-specific primer, which is specific for a first allele, including a first 5'-universal tail which comprises a binding site for a first universal FRET-based reporter primer (UFP)), and wherein said signal generating nucleic acid probe is configured, upon introduction of a target nucleic acid and exposure to appropriate conditions, to be degraded, and a signal is generated if said target nucleic acid is present in said mixture; and the presence or absence of said signal is configured to be detected (Para. [0012], oligonucleotides are provided that may be used to detect the amplification of a target nucleic acid and/or may be used to monitor or detect the amplification of an allele or polymorphism in a target nucleic acid. Such oligonucleotides are referred to herein as "universal FRET-based detector probes" the fluorescence may be further increased when the probe is digested by 5'-nuclease activity of polymerase; (Para. [0115], TaqMan R assays are typically carried out by performing nucleic acid amplification on a target polynucleotide using a nucleic acid polymerase having 5'-to-3' nuclease activity, a primer capable of hybridizing to the target polynucleotide, and an oligonucleotide probe.... Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification); Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein); and (C) instructions Para. [0023], The kits described herein may.... contain instructions or protocols for using Such reagents).

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Regarding Claim 153, Life Technologies discloses a system for determining the presence or absence of a target nucleic acid in the presence of a non-target nucleic acid (Abstract, The present disclosure is drawn to methods for detection, quantitation and analysis of nucleotides of interest, for example SNPs, in nucleic acid sequences of interest using universal FRET-based reporter primers; Para. [0047]. These systems, along with the many other systems available to the skilled artisan, may be suitable for use in polymerizing and/or amplifying target nucleic acids for use as described herein), said system comprising: (A) a reaction vessel (Para. [0050], amplifying comprises thermocycling using an instrument, for example, but not limited to, a GeneAmp(R) PCR System 9700, 9600, 2700 or 2400 thermocycler, an Applied Biosystems(R ViiATM 7 Real-Time PCR System, an Applied Biosystems(R 7500 Fast Real-Time PCR System, a 7900HT Fast Real-Time PCRSystem, and the like; Para. [0084], a plurality of reaction vessels can reside on the same Support. In some embodiments, lab-on-a-chip-like devices available, for example, from Caliper, Fluidigm and Life Technologies Corp., including the Ion 316TH and Ion 318TH Chip, may serve as reaction vessels in the disclosed methods), configured to receive: i. a sample; ii. a forward primer comprising a first region configured to hybridize to said target nucleic acid under amplification conditions and configured to not hybridize to said non-target nucleic acid under said amplification conditions, and a second region configured to not hybridize to said target nucleic acid under said amplification conditions; and iii. a signal generating nucleic acid probe, wherein said signal generating nucleic acid probe anneals to said second region, or regions complementary thereto, when subjected to said amplification conditions (Para. [0007], oligonucleotides are provided that are specific for a particular allele or polymorphism. Such oligonucleotides are referred to herein as allele-specific primers or ASPs. Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1). In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0021], the composition and/or reaction mixture includes one or more target nucleic acid molecules and a first allele-specific primer, which is specific for a first allele, including a first 5'-universal tail which includes a binding site for a first UFP, wherein the first UFP includes a first fluorophore and at least a first quencher moiety); (B) a thermocycler configured to subject said mixture to said amplification conditions, said amplification conditions appropriate to amplify said target nucleic acid with an amplification reaction (Para. [0050], amplifying comprises thermocycling using an instrument, for example, but not limited to, a GeneAmp(R) PCR System 9700, 9600, 2700 or 2400 thermocycler, an Applied Biosystems(R ViiATM 7 Real-Time PCR System, an Applied Biosystems(R 7500 Fast Real-Time PCR System, a 7900HT Fast Real-Time PCRSystem, and the like; Para. [0084], a plurality of reaction vessels can reside on the same Support. In some embodiments, lab-on-a-chip-like devices available, for example, from Caliper, Fluidigm and Life Technologies Corp., including the Ion 316TH and Ion 318TH Chip, may serve as reaction vessels in the disclosed methods), thereby amplifying said target nucleic acid such that said signal generating nucleic acid probe is degraded and a signal is generated if said target nucleic acid is present in said mixture; and (C) a detector configured to detect the presence or absence of said signal, thereby determining the presence or absence of said target nucleic acid in the presence of said non-target nucleic acid (Para. [0012], oligonucleotides are provided that may be used to detect the amplification of a target nucleic acid and/or may be used to monitor or detect the amplification of an allele or polymorphism in a target nucleic acid. Such oligonucleotides are referred to herein as "universal FRET-based detector probes" the fluorescence may be further increased when the probe is digested by 5'-nuclease activity of polymerase; (Para. [0115], TaqMan R assays are typically carried out by performing nucleic acid amplification on a target polynucleotide using a nucleic acid polymerase having 5'-to-3' nuclease activity, a primer capable of hybridizing to the target polynucleotide, and an oligonucleotide probe.... Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification); Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein).

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Regarding Claim 154, Life Technologies discloses a system, comprising a controller comprising or capable of accessing, computer readable media comprising non-transitory computer-executable instructions which, when executed by at least one electronic processor perform a method (Para. [0050], amplifying comprises thermocycling using an instrument, for example, but not limited to, a GeneAmp(R) PCR System 9700, 9600, 2700 or 2400 thermocycler, an Applied Biosystems(R) ViiATM 7 Real-Time PCR System, an Applied Biosystems(R) 7500 Fast Real-Time PCR System, a 7900HT Fast Real-Time PCR System, and the like (each comprising an integral computer executing programmed instructions); Para. [0084], a plurality of reaction vessels can reside on the same Support. In some embodiments, lab-on-a-chip-like devices available, for example, from Caliper, Fluidigm and Life Technologies Corp., including the Ion 316TH and Ion 318TH Chip, may serve as reaction vessels in the disclosed methods), comprising: (A) providing a sample comprising, or potentially comprising, said target nucleic acid and said non-target nucleic acid (Para. [0076], The target nucleic acid may be obtained from any Source, and may comprise any number of different compositional components.... target nucleic acids may be isolated from Samples using any of a variety of procedures known in the art); (B) forming a mixture comprising: i. said sample; ii. a forward primer comprising a first region configured to hybridize to said target nucleic acid under amplification conditions and configured to not hybridize to said non-target nucleic acid under said amplification conditions, and a second region configured to not hybridize to said target nucleic acid under said amplification conditions; and iii. a signal generating nucleic acid probe, wherein said signal generating nucleic acid probe anneals to said second region, or regions complementary thereto, when subjected to said amplification conditions (Para. [0007], oligonucleotides are provided that are specific for a particular allele or polymorphism. Such oligonucleotides are referred to herein as allele-specific primers or ASPs. Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1). In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is identical to the sequence of the universal FRET-based reporter primer. In certain embodiments, the 5'-universal tail includes a nucleotide sequence that is complementary to the sequence of the universal FRET-based reporter primer; Para. [0021], the composition and/or reaction mixture includes one or more target nucleic acid molecules and a first allele-specific primer, which is specific for a first allele, including a first 5'-universal tail which includes a binding site for a first UFP, wherein the first UFP includes a first fluorophore and at least a first quencher moiety); (C) subjecting said mixture to said amplification conditions, said amplification conditions appropriate to amplify said target nucleic acid with an amplification reaction, thereby amplifying said target nucleic acid such that said signal generating nucleic acid probe is degraded and a signal is generated if said target nucleic acid is present in said mixture; and (D) detecting the presence or absence of said signal, thereby determining the presence or absence of said target nucleic acid in the presence of said non-target nucleic acid (Para. [0012], oligonucleotides are provided that may be used to detect the amplification of a target nucleic acid and/or may be used to monitor or detect the amplification of an allele or polymorphism in a target nucleic acid. Such oligonucleotides are referred to herein as "universal FRET-based detector probes" the fluorescence may be further increased when the probe is digested by 5'-nuclease activity of polymerase; (Para. [0115], TaqMan R assays are typically carried out by performing nucleic acid amplification on a target polynucleotide using a nucleic acid polymerase having 5'-to-3' nuclease activity, a primer capable of hybridizing to the target polynucleotide, and an oligonucleotide probe.... Typically, the detectable label and quencher molecule are part of a single probe. As amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label (e.g., fluorescence) is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification); Para. [0116], detector probes include, but are not limited to, the 5'-exonuclease assay (TaqMan(R) probes described herein).

Claims 48, 113, 114, and 127 lack an inventive step under PCT Article 33(3) as being obvious over US 2014/0274774 A1 to Life Technologies Corporation (hereinafter Life Technologies) in view of The Secretary of State for the Home Department et al. (hereinafter Home Secretary of the UK).

Regarding Claim 48, Life Technologies discloses the method of claim 1. Life Technologies fails to explicitly disclose wherein said signal generating probe is configured to anneal to said second region and said first region. Home Secretary of the UK teaches a signal generating primer/probe configured to anneal to said second universal region and a first allele specific region (Pg. 26, Paras. 2-7. In the second stage of amplification, illustrated in Figure 3, a further specific amplification process is performed. It is much preferred that the second stage of amplification be conducted in the same vessel as the first, substantially simultaneous with the first amplification process. Such a possibility is described in more detail below. For this stage, an aliquot of the amplification products from the first stage, described above, are taken and contacted with a pair of "universal" forward primers and a "universal" reverse primer. These "universal" primers are described in more detail below. In step A, the strands 40 and 42 (copy strands which are equivalent to strands 14, 18 produced in the first stage as illustrated above) produced by the first stage 1 are denatured and contacted with the two "universal" forward primers 50, 52 and reverse "universal" primer 54. The two "universal" forward primers differ in terms of the 3' terminal end nucleotide 55 and in terms of a dye unit D or other form of label provided on the 5' end 56. The 3' end nucleotide 55 for the forward "universal" primers in this example is either C, "universal" primer 50, or G, "universal" primer 52. As the strands 40 and 42 represent the outcome of copies of copies of the originals being taken, unlike strands 14, 18, they both have tail portions 44, 46 respectively which arise from the copying of the "universal" primer portions of the locus specific forward primer and reverse primer in the first stage. The "universal" primers 50, 52 each have a sequence corresponding to the "universal" primer portion 34 of the first stage locus specific primers (second region) 30 up to location 38 of the locus specific forward primers 30. At location 55 the forward primers 50, 52 of the second stage have a base corresponding in identity to the identity of the nucleotide pairing to the SNP repeat in the stage 1 process (first region), in one case, and in the other case corresponding to the identity of the other option for the SNP repeat).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Life Technologies with the teaching of Home Secretary of the UK for the purpose of improving the specificity of SNP characterization, as taught by Home Secretary of the UK (Pg. 1, Para. 4. The present invention has amongst other aims a technique for improving SNP based investigations, particularly where the original sample levels are very small. Improvements in the specificity of the results and in terms of the ease with which a very large number of such SNPs can be investigated simultaneously are also considered).

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Regarding Claim 113, Life Technologies discloses the method of claim 104, wherein said signal generating nucleic acid probe is a target-specific probe comprising: (A) a sequence complementary or homologous to said 3' end of said universal tail; (B) a sequence complementary or homologous to said entire target-specific tail; OR (C) a sequence complementary or homologous to a portion of said first region (Para. [0032], FIGS. 7A through 7H: Performance comparison between certain of the UFP-based genotyping assays described herein and standard TaqMan(R) SNP assays (said standard assay comprising a labelled taqman probe complementary to an SNP target, i.e. only the first region). Allele discrimination plots from universal reporter-based genotyping assays were compared to TaqMan(R) SNP assays; Para. [0115], exemplary methods for polymerizing and/or amplifying and detecting nucleic acids suitable for use as described herein are commercially available as TaqMan(R) assays ...an oligonucleotide probe capable of hybridizing to said target polynucleotide 3' relative to the primer (i.e. to the first region). The oligonucleotide probe typically includes a detectable label (e.g., a fluorescent reporter molecule) and a quencher molecule capable of quenching the fluorescence of the reporter molecule; Para. [0106], An exemplary SNP assay where at least one universal FRET-based detector probe replaces the UFP is shown in FIG. 9 (the probe binds the tail region). An assay may include a pair of allele-specific primers where each allele specific primer includes a universal tail (ASP1 and ASP2), a shared locus-specific reverse primer (LSP), and a pair of universal detector probes for each of the two alleles (i.e. a unique probe for each allele)).

Life Technologies fails to explicitly disclose a probe complementary or homologous to said universal tail AND said allele specific first region.

Home Secretary of the UK teaches a signal generating primer/probe configured to anneal to a second universal region and a first allele specific region (Pg. 26, Paras. 2-7, In the second stage of amplification, illustrated in Figure 3, a further specific amplification process is performed. It is much preferred that the second stage of amplification be conducted in the same vessel as the first, substantially simultaneous with the first amplification process. Such a possibility is described in more detail below. For this stage, an aliquot of the amplification products from the first stage, described above, are taken and contacted with a pair of "universal" forward primers and a "universal" reverse primer. These "universal" primers are described in more detail below. In step A, the strands 40 and 42 (copy strands which are equivalent to strands 14, 18 produced in the first stage as illustrated above) produced by the first stage 1 are denatured and contacted with the two "universal" forward primers 50, 52 and reverse "universal" primer 54. The two "universal" forward primers differ in terms of the 3' terminal end nucleotide 55 and in terms of a dye unit D or other form of label provided on the 5' end 56. The 3' end nucleotide 55 for the forward "universal" primers in this example is either C, "universal" primer 50, or G, "universal" primer 52. As the strands 40 and 42 represent the outcome of copies of copies of the originals being taken, unlike strands 14, 18, they both have tail portions 44, 46 respectively which arise from the copying of the "universal" primer portions of the locus specific forward primer and reverse primer in the first stage. The "universal" primers 50, 52 each have a sequence corresponding to the "universal" primer portion 34 of the first stage locus specific primers (second region) 30 up to location 38 of the locus specific forward primers 30. At location 55 the forward primers 50, 52 of the second stage have a base corresponding in identity to the identity of the nucleotide pairing to the SNP repeat in the stage 1 process (first region), in one case, and in the other case corresponding to the identity of the other option for the SNP repeat).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Life Technologies with the teaching of Home Secretary of the UK for the purpose of improving the specificity of SNP characterization, as taught by Home Secretary of the UK (Pg. 1, Para. 4, The present invention has amongst other aims a technique for improving SNP based investigations, particularly where the original sample levels are very small. Improvements in the specificity of the results and in terms of the ease with which a very large number of such SNPs can be investigated simultaneously are also considered).

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/US2019/050050

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

Regarding Claim 114, Life Technologies discloses the method of claim 104, wherein said signal generating nucleic acid probe is a target-specific probe comprising: (A) a sequence complementary or homologous to said 3' end of said universal tail; OR (B) a sequence complementary or homologous to a portion of the target-specific tail (Para. [0032], FIGS. 7A through 7H: Performance comparison between certain of the UFP-based genotyping assays described herein and standard TaqMan(R) SNP assays (said standard assay comprising a labelled taqman probe complementary to an SNP target, i.e. only the first region). Allele discrimination plots from universal reporter-based genotyping assays were compared to TaqMan(R) SNP assays; Para. [0115], exemplary methods for polymerizing and/or amplifying and detecting nucleic acids suitable for use as described herein are commercially available as TaqMan(R) assays ...an oligonucleotide probe capable of hybridizing to said target polynucleotide 3' relative to the primer (i.e. to the first region). The oligonucleotide probe typically includes a detectable label (e.g., a fluorescent reporter molecule) and a quencher molecule capable of quenching the fluorescence of the reporter molecule; Para. [0106]. An exemplary SNP assay where at least one universal FRET-based detector probe replaces the UFP is shown in FIG. 9 (the probe binds the tail region). An assay may include a pair of allele-specific primers where each allele specific primer includes a universal tail (ASP1 and ASP2), a shared locus-specific reverse primer (LSP), and a pair of universal detector probes for each of the two alleles (i.e. a unique probe for each allele)). Life Technologies fails to explicitly disclose a probe complementary or homologous to said universal tail AND said allele specific first region.

Home Secretary of the UK teaches a signal generating primer/probe configured to anneal to a second universal region and a first allele specific region (Pg. 26, Paras. 2-7, in the second stage of amplification, illustrated in Figure 3, a further specific amplification process is performed. It is much preferred that the second stage of amplification be conducted in the same vessel as the first, substantially simultaneous with the first amplification process. Such a possibility is described in more detail below. For this stage, an aliquot of the amplification products from the first stage, described above, are taken and contacted with a pair of "universal" forward primers and a "universal" reverse primer. These "universal" primers are described in more detail below. In step A, the strands 40 and 42 (copy strands which are equivalent to strands 14, 18 produced in the first stage as illustrated above) produced by the first stage 1 are denaturated and contacted with the two "universal" forward primers 50, 52 and reverse "universal" primer 54. The two "universal" forward primers differ in terms of the 3' terminal end nucleotide 55 and in terms of a dye unit D or other form of label provided on the 5' end 56. The 3' end nucleotide 55 for the forward "universal" primers in this example is either C, "universal" primer 50, or G, "universal" primer 52. As the strands 40 and 42 represent the outcome of copies of copies of the originals being taken, unlike strands 14, 18, they both have tail portions 44, 46 respectively which arise from the copying of the "universal" primer portions of the locus specific forward primer and reverse primer in the first stage. The "universal" primers 50, 52 each have a sequence corresponding to the "universal" primer portion 34 of the first stage locus specific primers (second region) 30 up to location 38 of the locus specific forward primers 30. At location 55 the forward primers 50, 52 of the second stage have a base corresponding in identity to the identity of the nucleotide pairing to the SNP repeat in the stage 1 process (first region), in one case, and in the other case corresponding to the identity of the other option for the SNP repeat).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Life Technologies with the teaching of Home Secretary of the UK for the purpose of improving the specificity of SNP characterization, as taught by Home Secretary of the UK (Pg. 1, Para. 4, The present invention has amongst other aims a technique for improving SNP based investigations, particularly where the original sample levels are very small. Improvements in the specificity of the results and in terms of the ease with which a very large number of such SNPs can be investigated simultaneously are also considered).

Regarding Claim 127, Life Technologies discloses the method of claim 73. Life Technologies fails to explicitly disclose wherein said signal generating probe is configured to anneal to said second region and said first region.

Home Secretary of the UK teaches a signal generating primer/probe configured to anneal to a second universal region and a first allele specific region (Pg. 26, Paras. 2-7, in the second stage of amplification, illustrated in Figure 3, a further specific amplification process is performed. It is much preferred that the second stage of amplification be conducted in the same vessel as the first, substantially simultaneous with the first amplification process. Such a possibility is described in more detail below. For this stage, an aliquot of the amplification products from the first stage, described above, are taken and contacted with a pair of "universal" forward primers and a "universal" reverse primer. These "universal" primers are described in more detail below. In step A, the strands 40 and 42 (copy strands which are equivalent to strands 14, 18 produced in the first stage as illustrated above) produced by the first stage 1 are denaturated and contacted with the two "universal" forward primers 50, 52 and reverse "universal" primer 54. The two "universal" forward primers differ in terms of the 3' terminal end nucleotide 55 and in terms of a dye unit D or other form of label provided on the 5' end 56. The 3' end nucleotide 55 for the forward "universal" primers in this example is either C, "universal" primer 50, or G, "universal" primer 52. As the strands 40 and 42 represent the outcome of copies of copies of the originals being taken, unlike strands 14, 18, they both have tail portions 44, 46 respectively which arise from the copying of the "universal" primer portions of the locus specific forward primer and reverse primer in the first stage. The "universal" primers 50, 52 each have a sequence corresponding to the "universal" primer portion 34 of the first stage locus specific primers (second region) 30 up to location 38 of the locus specific forward primers 30. At location 55 the forward primers 50, 52 of the second stage have a base corresponding in identity to the identity of the nucleotide pairing to the SNP repeat in the stage 1 process (first region), in one case, and in the other case corresponding to the identity of the other option for the SNP repeat).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Life Technologies with the teaching of Home Secretary of the UK for the purpose of improving the specificity of SNP characterization, as taught by Home Secretary of the UK (Pg. 1, Para. 4, The present invention has amongst other aims a technique for improving SNP based investigations, particularly where the original sample levels are very small. Improvements in the specificity of the results and in terms of the ease with which a very large number of such SNPs can be investigated simultaneously are also considered).

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/US2019/050050

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

Claims 69, 76, 80, and 148 lack an inventive step under PCT Article 33(3) as being obvious over US 2014/0274774 A1 to Life Technologies Corporation (hereinafter Life Technologies) in view of US 2017/0362636 A1 to California Institute of Technology (hereinafter CalTech).

Regarding Claim 69, Life Technologies discloses the method of claim 63, wherein said second universal probe binding motif or said first universal probe binding motif are on the 5' end of said first region and are on the 3' end of said universal primer binding motif (Para. [0007], oligonucleotides are provided that are specific for a particular allele or polymorphism. Such oligonucleotides are referred to herein as allele-specific primers or ASPs. Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1); Para. [0115], a primer capable of hybridizing to the target polynucleotide, and an oligonucleotide probe capable of hybridizing to said target polynucleotide 3' relative to the primer. The oligonucleotide probe typically includes a detectable label (e.g., a fluorescent reporter molecule) and a quencher molecule capable of quenching the fluorescence of the reporter molecule).

Life Technologies fails to explicitly disclose wherein said second universal probe binding motif is adjacent to said first universal probe binding motif.

CalTech teaches detection of nucleic acid amplification (Abstract, The present invention relates to methods of nucleic acid analyte detection by PCR) using first and second signal generating probes that bind to adjacent probe binding motifs (Para. [0155], 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). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Life Technologies with the teaching of CalTech for the purpose of using FRET based detection, as taught by CalTech.

Regarding Claim 76, Life Technologies discloses the method of claim 73. Life Technologies fails to explicitly disclose wherein said target-specific RNA base, DNA base, or unnatural base facilitates the selective amplification of said target nucleic acid.

CalTech teaches a target specific RNA base in a hybrid primer facilitates the selective amplification of a target nucleic acid (Para. [0114], The composite amplification primers are primers that are composed of RNA and DNA portions. In the amplification composite primer, both the RNA and the DNA portions are generally complementary and can hybridize to a sequence in the amplification-ready product to be copied or amplified.... The 5'-RNA portion of this primer extension product in a RNA/DNA heteroduplex is susceptible to cleavage by RNase H, thus freeing a portion of the polynucleotide to the hybridization of an additional composite amplification primer. The extension of the amplification composite primer by a DNA polymerase with strand displacement activity releases the primer extension product from the original primer and creates another copy of the sequence of the polynucleotide. Repeated rounds of primer hybridization, primer extension with strand displacement DNA synthesis, and RNA cleavage create multiple copies of the strand-specific sequence of the polynucleotide).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Life Technologies with the teaching of CalTech for the purpose of selectively amplifying a DNA target strand, as taught by CalTech.

Regarding Claim 80, Life Technologies discloses the method of claim 79. Life Technologies fails to explicitly disclose wherein said additional enzyme is ribonuclease H.

CalTech teaches an additional enzyme for target amplification is ribonuclease H (Para. [0114], The composite amplification primers are primers that are composed of RNA and DNA portions. In the amplification composite primer, both the RNA and the DNA portions are generally complementary and can hybridize to a sequence in the amplification-ready product to be copied or amplified.... The 5'-RNA portion of this primer extension product in a RNA/DNA heteroduplex is susceptible to cleavage by RNase H, thus freeing a portion of the polynucleotide to the hybridization of an additional composite amplification primer. The extension of the amplification composite primer by a DNA polymerase with strand displacement activity releases the primer extension product from the original primer and creates another copy of the sequence of the polynucleotide. Repeated rounds of primer hybridization, primer extension with strand displacement DNA synthesis, and RNA cleavage create multiple copies of the strand-specific sequence of the polynucleotide).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Life Technologies with the teaching of CalTech for the purpose of selectively amplifying a DNA target strand, as taught by CalTech.

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/US2019/050050

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

Regarding Claim 148, Life Technologies discloses the method of claim 143, wherein said second universal probe binding motif or said first universal probe binding motif are on the 5' end of said first region and are on the 3' end of said universal primer binding motif (Para. [0007], oligonucleotides are provided that are specific for a particular allele or polymorphism. Such oligonucleotides are referred to herein as allele-specific primers or ASPs. Such allele-specific primers include an allele-specific portion and a 5'-universal tail; wherein the 5'-universal tail includes a binding site for a universal FRET based reporter primer (herein referred to as "UFP") and the universal tail is not complementary to the target nucleic acid sequence (see FIG. 1); Para. [0115], a primer capable of hybridizing to the target polynucleotide, and an oligonucleotide probe capable of hybridizing to said target polynucleotide 3' relative to the primer. The oligonucleotide probe typically includes a detectable label (e.g., a fluorescent reporter molecule) and a quencher molecule capable of quenching the fluorescence of the reporter molecule).

Life Technologies fails to explicitly disclose wherein said second universal probe binding motif is adjacent to said first universal probe binding motif.

CalTech teaches detection of nucleic acid amplification (Abstract, The present invention relates to methods of nucleic acid analyte detection by PCR) using first and second signal generating probes that bind to adjacent probe binding motifs (Para. [0155], 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 use 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). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Life Technologies with the teaching of CalTech for the purpose of using FRET based detection, as taught by CalTech.

Claims 1-23, 25-102, and 104-154 meet the criteria set out in PCT Article 33(4), and thus have industrial applicability because the subject matter claimed can be made or used in industry.

CIT/38075-716.601
WM/212/ADCO/DBA/ALT/SPEL

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT AND
THE WRITTEN OPINION OF THE INTERNATIONAL
SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

To:

ZAKI MEGEED
WILSON SONSINI GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

Date of mailing
(day/month/year)

12 APR 2013

Applicant's or agent's file reference
38075-716601

FOR FURTHER ACTION See paragraphs 1 and 4 below

International application No.
PCT/US 13/24509

International filing date
(day/month/year) 01 February 2013 (01.02.2013)

Applicant CALIFORNIA INSTITUTE OF TECHNOLOGY

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

Filing of amendments and statement under Article 19:

The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

Where? Directly to the International Bureau of WIPO, 34 chemin des Colombettes
1211 Geneva 20, Switzerland, Facsimile No.: +41 22 338 82 70

For more detailed instructions, see *PCT Applicant's Guide*, International Phase, paragraphs 9.004 - 9.011.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. ☐ With regard to any protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

☐ the protest together with the decision thereon has been transmitted to the International Bureau together with any request to forward the texts of both the protest and the decision thereon to the designated Offices.

☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established. Following the expiration of 30 months from the priority date, these comments will also be made available to the public.

Shortly after the expiration of **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before the completion of the technical preparations for international publication (Rules 90bis.1 and 90bis.3).

Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months.

For details about the applicable time limits, Office by Office, see www.wipo.int/pct/en/texts/time_limits.html and the *PCT Applicant's Guide*, National Chapters.

Name and mailing address of the ISA/
Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-3201

Authorized officer

Lee W. Young

PCT Helpdesk: 571-272-4900

Telephone No. PCT.OSP: 571-272-3774

WSGR PATENT DOCKET

U.S. 101 105 FOREIGN: ☒

DOCKETED: 4-15-13 BY: [signature]

ACTION: Amend Claims 1012-13

DUE DATES: Written Opinion 12-3-13

ATTY C/M #

RECEIVED
APR 15 2013

WILSON SONSINI
GOODRICH & ROSATI

Form PCT/ISA/220 (July 2010)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 38075-716601	FOR FURTHER ACTION		see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. PCT/US 13/24509	International filing date (day/month/year) 01 February 2013 (01.02.2013)	(Earliest) Priority Date (day/month/year) 03 February 2012 (03.02.2012)	
Applicant CALIFORNIA INSTITUTE OF TECHNOLOGY			

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 2 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the **language**, the international search was carried out on the basis of:

- ☒ the international application in the language in which it was filed.
☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6bis(a)).

c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☐ **Certain claims were found unsearchable** (see Box No. II).

3. ☐ **Unity of invention is lacking** (see Box No. III).

4. With regard to the **title**,

- ☒ the text is approved as submitted by the applicant.
☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

- ☒ the text is approved as submitted by the applicant.
☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the **drawings**,

- a. the figure of the **drawings** to be published with the abstract is Figure No. _____
☐ as suggested by the applicant.
☐ as selected by this Authority, because the applicant failed to suggest a figure.
☐ as selected by this Authority, because this figure better characterizes the invention.
- b. ☒ none of the figures is to be published with the abstract.

Form PCT/ISA/210 (first sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/24509

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/68 (2013.01)

USPC - 435/6.1

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)

USPC 435/6.1

IPC C12Q 1/68 (2013.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC 435/6.11; 435/6.19 (Text search - see search terms below)Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Scholar Search Terms: signal, encoding, decoding, multiplexed, biochemical, assays

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2010/0267064 A1 (KARTALOV et al) 21 October 2010 (21.10.2010) entire document	1-74
A	US 2005/0260640 A1 (ANDERSEN et al) 24 November 2005 (24.11.2005) entire document	1-74
A	US 2007/0231824 A1 (CHEE et al) 04 October 2007 (04.10.2007) entire document	1-74
A	US 2002/0022273 A1 (EMPEDOCLES et al) 21 February 2002 (21.02.2002) entire document	1-74
A	US'2010/0210472 A1 (EMPEDOCLES et al) 19 August 2010 (19.08.2010) entire document	1-74
A	US 2008/0069733 A1 (MALTEZOS et al) 20 March 2008 (20.03.2008) entire document	1-74
A	US 2009/0062129 A1 (McKERNAN et al) 05 March 2009 (05.03.2009) entire document	1-74
A	US 2011/0237459 A1 (NOVA et al) 29 September 2011 (29.09.2011) entire document	1-74
A	US 2005/0214753 A1 (SCHULTZ et al) 29 September 2005 (29.09.2005) entire document	1-74
A	US 2005/0164264 A1 (SHIPWASH) 28 July 2005 (28.07.2005) entire document	1-74

☐ Further documents are listed in the continuation of Box C.

* 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 another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

22 March 2013 (22.03.2013)

Date of mailing of the international search report

12 APR 2013

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

IPR2024-01451

California Institute of Technology - Ex. 2003 Page 172

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: ZAKI MEGEED
WILSON SONSINI GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)

Date of mailing
(day/month/year)

12 APR 2013

Applicant's or agent's file reference
38075-716601

FOR FURTHER ACTION

See paragraph 2 below

International application No.
PCT/US 13/24509

International filing date (day/month/year)
01 February 2013 (01.02.2013)

Priority date (day/month/year)
03 February 2012 (03.02.2012)

International Patent Classification (IPC) or both national classification and IPC
IPC(8) - C12Q 1/68 (2013.01)
USPC - 435/6.1

Applicant CALIFORNIA INSTITUTE OF TECHNOLOGY

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1 bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-3201

Date of completion of this opinion
22 March 2013 (22.03.2013)

Authorized officer:
Lee W. Young
PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (July 2011)

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 13/24509

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:
 - ☒ the international application in the language in which it was filed.
 - ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).
2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43bis.1(a))
3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing filed or furnished:
 - a. (means)
 - ☐ on paper
 - ☐ in electronic form
 - b. (time)
 - ☐ in the international application as filed
 - ☐ together with the international application in electronic form
 - ☐ subsequently to this Authority for the purposes of search
4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
5. Additional comments:

Form PCT/ISA/237 (Box No. I) (July 2011)

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/US 13/24509

Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-74	YES
	Claims	NONE	NO
Inventive step (IS)	Claims	1-74	YES
	Claims	NONE	NO
Industrial applicability (IA)	Claims	1-74	YES
	Claims	NONE	NO

2. Citations and explanations:

Claims 1-48 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest an assay that is 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.

Regarding claim 1-48, all comprising similar subject matter, there is no prior art of record that discloses an assay that is 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. The closest prior art, US 2010/0267064 A1 to Kartalov et al. (hereinafter "Kartalov"), discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092] and immobilization (para. [0112]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the assay as described above.

Claims 49-54 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising 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; providing a sample comprising, or potentially comprising, at least one of said analytes; 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; cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

Regarding claims 49-54, all comprising similar subject matter, there is no prior art of record that discloses a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising 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; providing a sample comprising, or potentially comprising, at least one of said analytes; 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; cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme. The closest prior art, Kartalov, discloses a reporting scheme and intensity and/or spectrum of an optical signal (para. [0080]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the method as described above.

----- CONTINUED IN SUPPLEMENTAL BOX -----

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.

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Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:
-Box V-

Claims 55-60 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising: 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; providing a sample comprising, or potentially comprising, at least one of said analytes; 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; cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and 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.

Regarding claims 55-60, all comprising similar subject matter, there is no prior art of record that discloses a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising: 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; providing a sample comprising, or potentially comprising, at least one of said analytes; 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; cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and 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. The closest prior art, Kartalov, discloses a reporting scheme, tuning and detuning (para. [0077]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the method as described above.

Claims 61-64 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a non-degenerate coding scheme capable of unambiguously encoding the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, or melting curve analysis.

Regarding claims 61-64, all comprising similar subject matter, there is no prior art of record that discloses a non-degenerate coding scheme capable of unambiguously encoding the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, or melting curve analysis. The closest prior art, Kartalov, discloses a reporting scheme, tuning and detuning (para. [0077]). There is nothing of record to lead one of ordinary skill in the art to modify the reporting scheme as taught by Kartalov to arrive at the non-degenerate coding scheme as described above.

Claim 65 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a system for detecting the presence or absence of each analyte of a plurality of analytes, comprising:

- 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;
- providing a sample comprising, or potentially comprising, at least one of said analytes;
- 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;
- cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and
- determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

Regarding claim 65, there is no prior art of record that discloses a system for detecting the presence or absence of each analyte of a plurality of analytes, comprising:

- 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;
- providing a sample comprising, or potentially comprising, at least one of said analytes;
- 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;
- cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and
- determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092] and immobilization (para. [0112])). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the system as described above.

-Continued on following Supplemental Box-

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 13/24509

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

-Continued from previous Supplemental Box-

Claim 66 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a system 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 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.

Regarding claim 66, there is no prior art of record that discloses a system 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 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. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092] and immobilization (para. [0112]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the system as described above.

-Continued in the following Supplemental Box-

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 13/24509

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

-Continued from previous Supplemental Box-

Claim 67 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a method of detecting the presence or absence of each analyte of a plurality of analytes, 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.

Regarding claim 67, there is no prior art of record that discloses a method of detecting the presence or absence of each analyte of a plurality of analytes, 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. The closest prior art, Kartalov, discloses five analytes (para. [0156]), 6 samples and combinations thereof (para. [0092]) and immobilization (para. [0112]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the system as described above.

-Continued in following Supplemental Box-

WRITTEN OPINION OF THE
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Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

-Continued from previous Supplemental Box-

Claim 68 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a method of detecting the presence or absence of each analyte of a plurality of analytes,

comprising:

- g. obtaining the identity of each of said analytes from a party;
- h. 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;
- 1. 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
- j. obtaining said cumulative measurement from said party;
- k. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and
- 1. providing said party with information about the presence or absence of each of said analytes.

Regarding claim 68, there is no prior art of record that discloses a method of detecting the presence or absence of each analyte of a plurality of analytes,

comprising:

- g. obtaining the identity of each of said analytes from a party;
- h. 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;
- 1. 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
- j. obtaining said cumulative measurement from said party;
- k. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and
- 1. providing said party with information about the presence or absence of each of said analytes. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092] and immobilization (para. [0112])). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the method as described above.

-Continued in following Supplemental Box-

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US 13/24509

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

-Continued from previous Supplemental Box-

Claim 69 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a composition for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising a first value, wherein said reagents are capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, or melting curve analysis.

Regarding claim 69, there is no prior art of record that discloses a composition for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising a first value, wherein said reagents are capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, or melting curve analysis. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092] and immobilization (para. [0112])). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the composition as described above.

Claim 70 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a composition 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.

Regarding claim 70, there is no prior art of record that discloses a composition 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. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092] and immobilization (para. [0112])). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the composition as described above.

Claim 71 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a kit 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, or melting curve analysis.

Regarding claim 71, there is no prior art of record that discloses a kit 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, or melting curve analysis. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092] and immobilization (para. [0112])). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the kit as described above.

Claim 72 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a kit 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.

Regarding claim 72, there is no prior art of record that discloses a kit 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. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092] and immobilization (para. [0112])). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the kit as described above.

-Continued in following Supplemental Box-

WRITTEN OPINION OF THE
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International application No.
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Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

-Continued from previous Supplemental Box-

Claim 73 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a kit 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, or melting curve analysis.

Regarding claim 73, there is no prior art of record that discloses a kit 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, or melting curve analysis. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092]) and immobilization (para. [0112]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the kit as described above.

Claim 74 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a kit for detecting the presence or absence of each analyte of a plurality of analytes, a kit body (601), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (602), a bottle comprising primers for amplification (603), and a bottle comprising reagents for amplification (604), each probe generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said probes generates only one second value.

Regarding claim 74, there is no prior art of record that discloses a kit for detecting the presence or absence of each analyte of a plurality of analytes, a kit body (601), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (602), a bottle comprising primers for amplification (603), and a bottle comprising reagents for amplification (604), each probe generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said probes generates only one second value. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092]) and immobilization (para. [0112]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the kit as described above.

Claims 1-74 have industrial applicability as defined by PCT Article 33(4) because the subject matter may be made or used in industry.

SEARCH HISTORY

Application Number	PCT/US 13/24509
Search Conducted By	JCG
Search Approved By	SV

US/IPC Classifications Searched	435/6.1; C12Q 1/68 (2013.01)
Date Conducted	[22 March 2013 (22.03.2013)]

Documentation Searched	435/6.1,6.11,6.19
Search Terms Used	signal, encoding, decoding, multiplexed, biochemical, assays
Date Conducted	[22 March 2013 (22.03.2013)]

Electronic Database Searched	PatBase
Files Searched	Full-text: AU BE BR CA CH CN DE DK EP ES FI FR GB IN JP KR SE TH TW US WO Bibliographic: (Europe) AT BA BE BG CH CS CY CZ DD DK EE ES FI GE GR HR HU IE IS IT LT LU LV MC MD MT NL NO PL PT RO RS SE SI SK SM TR UA YU (Asia) EA GC HK ID IL IN KZ MN MY PH RU SG SU TH TJ TW UZ VN (North America) CA CR CU DO GT HN MX NI PA SV TT (South America) AR BR CL CO EC PE UY (Australasia) AU NZ (Africa) AP DZ EG KE MA MW OA ZA ZM ZW
Date Conducted	[22 March 2013 (22.03.2013)]
Search Logic:	

- 1) UC=(435/6.1 or 435/6.1 or 435/6.11 or 435/6.19) (15067)
- 2) IC=(C12Q1/68) (90142)
- 3) PA=(california institute of technology) (2284)
- 4) 3 and FT=(multiplex#) (363)

5) 4 and FT=(analyte? or detect or presence or absence not immobilize# not separate# not "mass spectrometry" not "melting curve") (19)

6) (1 or 2) and 5 (1)
 7) (1 or 2) and FT=(multiplex#) (11237)
 8) 7 and FT=(intensity# or luminosity# or bright# or fluorescence?) (6909)
 9) 7 and SP=((three or "3") and (intensity# or luminosity# or bright# or fluorescence?)) (3983)
 10) 7 and SP=((three or "3" OR=3:100) and (intensity# or luminosity# or bright# or fluorescence?)) (5457)
 11) (8 or 9 or 10) and FT=(analyte?) (1543)
 12) 10 and FT=(analyte?) (1301)
 13) (11 or 12) and FT=(code# or coding) (1032)
 14) 12 and FT=(code# or coding) (886)
 15) (13 or 14) and FT=(wavelength# or wavelength?) (717)
 16) 14 and FT=(wavelength# or wavelength?) (624)
 17) 15 and sp=((("5" or "6" or "7" or "8" or "9" or "10") or (five or six or seven or eight or nine or ten)) and (wavelength?)) (292)
 18) 16 and sp=((("5" or "6" or "7" or "8" or "9" or "10") or (five or six or seven or eight or nine or ten)) and (wavelength?)) (272)
 19) 17 and FT=(electromagnet#) (18)
 20) 18 and SP=(signal? and (fluorescence? or fluorescent)) (157)
 21) 18 and DSC=(fluorescence? or fluorescent) (266)
 22) 17 and FT=(lyophilize# or lyophilized) (51)
 23) 21 and FT=(lyophilize# or lyophilized) (46)
 24) 21 and FT=(hybridize# and probe?) (206)
 25) 24 and SP=(complement# and primer? and probe?) (32)
 26) 24 and FT=(PCR or "polymerase chain reaction") (190)
 27) 26 and SP=((PCR or "polymerase chain reaction") and ("end?point" or "real?time" or "RT?PCR" or "digital")) (120)
 28) 27 and FT=(chromatogram?) (10)
 29) 26 and FT=(chromatogram?) (16)
 30) 29 and SP=(chromatogram? and positive and control?) (0)
 31) 27 and FT=(polynucleotide?) (70)
 32) 26 and FT=(polynucleotide?) (112)
 33) (31 or 32) and SP=((polynucleotide?) and (animal or plant or bacteria or fungus or parasite or virus)) (26)
 34) 31 and ft=("hiv" or "human immunodeficiency virus" or "herpes simplex virus" or "human papilloma virus" or "hpv") (41)
 35) 32 and ft=("hiv" or "human immunodeficiency virus" or "herpes simplex virus" or "human papilloma virus" or "hpv") (68)
 36) 33 and ft=("hiv" or "human immunodeficiency virus" or "herpes simplex virus" or "human papilloma virus" or "hpv") (13)
 37) 34 and ft=(poly?protease or "p17" or "e6" or "e7") (11)
 38) 13 and FT=(non?degenerate#) (8)
 39) 13 and FT=(coding NOT degenerate NOT degeneracy) (466)
 40) 39 and 37 (4)
 41) PN=(WO2005094532) (1)
 42) PN=(US8137626) (1)
 43) CTF 42 (20)
 44) 43 and FT=(multiplex) (2)
 45) CTB 42 (50)
 46) 45 and FT=(multiplex) (11)

- 47) PN=(US8187541) (1)
48) PN=(EP2267451) (1)
49) FT=(multiplex and intensities and wavelengths) (2190)
50) 49 and SP=(intensities and 3:100) (897)
51) 50 and SP=(wavelengths and 5:100) (447)
52) 51 and SP=((code# or coding) and wavelengths and intensities) (37)

Electronic Database Searched

Google Scholar

Files Searched	
Date Conducted	[22 March 2013 (22.03.2013)]
Search Logic:	
SIGNAL ENCODING AND DECODING MULTIPLEXED BIOCHEMICAL ASSAYS "California institute of technology" coding scheme (three and intensities) and (five and wavelengths)	

Electronic Database Searched	THOMSON INNOVATION
Files Searched	
Date Conducted	29 March 2013 (29.03.2013)
Search Logic:	

9 9 US Grant, CA
 Grant, US App, (((ALL=(assay and
 CA App, WO (immobilization or separation
 App, FR App, or mass ADJ spectrometry or
 EP Grant, DE melting ADJ curve ADJ
 Util, EP App, analysis))) AND ALL=(sample
 DE Grant, GB ADJ volume)) AND
 App, DE App, (ALL=(without near5
 JP App, KR (immobilization or separation
 Grant, KR App, or mass ADJ spectrometry or
 Other, DWPI, JP melting ADJ curve ADJ
 Util, KR Util, analysis)))) AND
 CN Util, JP (ALL=(single ADJ sample)))
 Grant, CN AND (ALL=(without ADJ
 Grant, CN App, immobilization))) AND
 VN Grant, MY (ALL=(encoding));
 Grant, TH
 Grant, VN App

8 9 US Grant, CA Grant, US App,
 CA App, WO App, FR App, (((ALL=(assay and
 EP Grant, DE Util, EP App, (immobilization or separation
 DE Grant, GB App, DE App, or mass ADJ spectrometry or

	JP App, KR Grant, KR App, Other, DWPI, JP Util, KR Util , CN Util, JP Grant, CN Grant, CN App, VN Grant, MY Grant, TH Grant, VN App	melting ADJ curve ADJ analysis)) AND ALL=(sample ADJ volume)) AND (ALL=(without near5 (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)))) AND (ALL=(single ADJ sample))) AND (ALL=(without ADJ immobilization));
7 0	US Grant, CA Grant, US App, CA App, WO App, FR App, EP Grant, DE Util, EP App, DE Grant, GB App, DE App, JP App, KR Grant, KR App, Other, DWPI, JP Util, KR Util , CN Util, JP Grant, CN Grant, CN App, VN Grant, MY Grant, TH Grant, VN App	((((ALL=(assay and (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)) AND ALL=(sample ADJ volume)) AND (ALL=(without near5 (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)))) AND (ALL=(single ADJ sample))) AND (ALL=(without ADJ melting ADJ curve ADJ analysis)));
6 0	US Grant, CA Grant, US App, CA App, WO App, FR App, EP Grant, DE Util, EP App, DE Grant, GB App, DE App, JP App, KR Grant, KR App, Other, DWPI, JP Util, KR Util , CN Util, JP Grant, CN Grant, CN App, VN Grant, MY Grant, TH Grant, VN App	((((ALL=(assay and (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)) AND ALL=(sample ADJ volume)) AND (ALL=(without near5 (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)))) AND (ALL=(single ADJ sample))) AND (ALL=(without ADJ mass ADJ spectrometry)));
5 204	US Grant, CA Grant, US App, CA App, WO App, FR App, EP Grant, DE Util, EP App, DE Grant, GB App, DE App, JP App, KR Grant, KR App,	((ALL=(assay and (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ

	Other, DWPI, JP Util, KR Util , CN Util, JP Grant, CN Grant, CN App, VN Grant, MY Grant, TH Grant, VN App	analysis)) AND ALL=(sample ADJ volume)) AND (ALL=(without near5 (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)))) AND (ALL=(single ADJ sample));
4 0	US Grant, CA Grant, US App, CA App, WO App, FR App, EP Grant, DE Util, EP App, DE Grant, GB App, DE App, JP App, KR Grant, KR App, Other, DWPI, JP Util, KR Util , CN Util, JP Grant, CN Grant, CN App, VN Grant, MY Grant, TH Grant, VN App	((((ALL=(assay and (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)) AND ALL=(sample ADJ volume)) AND (ALL=(without near5 (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)))) AND (ALL=(without near5 (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis) same assay)))) AND (ALL=(single ADJ sample ADJ volume));
3 296	US Grant, CA Grant, US App, CA App, WO App, FR App, EP Grant, DE Util, EP App, DE Grant, GB App, DE App, JP App, KR Grant, KR App, Other, DWPI, JP Util, KR Util , CN Util, JP Grant, CN Grant, CN App, VN Grant, MY Grant, TH Grant, VN App	((ALL=(assay and (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)) AND ALL=(sample ADJ volume)) AND (ALL=(without near5 (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)))) AND (ALL=(without near5 (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis) same assay));
2 1300	US Grant, CA Grant, US App, CA App, WO App, FR App, EP Grant, DE Util, EP App,	(ALL=(assay and (immobilization or separation

DE Grant, GB App, DE App,
JP App, KR Grant, KR App,
Other, DWPI, JP Util, KR Util
, CN Util, JP Grant, CN Grant,
CN App, VN Grant, MY
Grant, TH Grant, VN App

or mass ADJ spectrometry or
melting ADJ curve ADJ
analysis)) AND
ALL=(sample ADJ volume))
AND (ALL=(without near5
(immobilization or separation
or mass ADJ spectrometry or
melting ADJ curve ADJ
analysis))));

1 17526

US Grant, CA Grant, US App,
CA App, WO App, FR App,
EP Grant, DE Util, EP App,
DE Grant, GB App, DE App,
JP App, KR Grant, KR App,
Other, DWPI, JP Util, KR Util
, CN Util, JP Grant, CN Grant,
CN App, VN Grant, MY
Grant, TH Grant, VN App

ALL=(assay and
(immobilization or separation
or mass ADJ spectrometry or
melting ADJ curve ADJ
analysis)) AND
ALL=(sample ADJ volume);

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference P1272-PCT	FOR FURTHER ACTION see Form PCT/ISA/220 as well as, where applicable, item 5 below.	
International application No. PCT/US2013/053512	International filing date (<i>day/month/year</i>) 02 August 2013 (02.08.2013)	(Earliest) Priority Date (<i>day/month/year</i>) 03 August 2012 (03.08.2012)
Applicant CALIFORNIA INSTITUTE OF TECHNOLOGY		

This International search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of :

- ☒ the international application in the language in which it was filed
☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))

b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6bis(a)).

c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☒ **Certain claims were found unsearchable** (See Box No. II)

3. ☐ **Unity of invention is lacking** (See Box No. III)

4. With regard to the **title**,

- ☒ the text is approved as submitted by the applicant.
☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

- ☒ the text is approved as submitted by the applicant.
☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the drawings,

- a. the figure of the **drawings** to be published with the abstract is Figure No. 3
☒ as suggested by the applicant.
☐ as selected by this Authority, because the applicant failed to suggest a figure.
☐ as selected by this Authority, because this figure better characterizes the invention.
b. ☐ none of the figure is to be published with the abstract.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/053512

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.: 14-17,23-26
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:
Claims 14-17 and 23-26 are unclear since they are referring to the multiple dependent claims which do not comply with PCT Rule 6.4(a).
3. ☒ Claims Nos.: 13,20-22,27-32
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 additional 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.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/053512**A. CLASSIFICATION OF SUBJECT MATTER****C12Q 1/68(2006.01)i, C12M 1/38(2006.01)i, C12N 15/11(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; C12M 1/00; G06F 19/00; G01N 21/64; C12P 19/34; G06F 19/10; C12M 1/34; C12M 1/38; C12N 15/11

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: amplicons, multiplexed amplification, single channel detector, sum amplitude signal, processor-based hardware analyzer

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FU et al., 'Multiplex detection and SNP genotyping in a single fluorescence channel' PLoS One, Vol.7, Issue 1, Article No.e30340, pp.1-8 (January 2012) See the whole document.	1 12, 18 19, 33 39
A	US 2011-0223602 A1 (WHITMAN et al.) 15 September 2011 See the whole document.	1-12, 18-19, 33-39
A	US 2010-0324834 A1 (TREPTOW et al.) 23 December 2010 See the whole document.	1-12, 18-19, 33-39
A	US 2012-0040352 A1 (WANGH et al.) 16 February 2012 See the whole document.	1-12, 18-19, 33-39
A	US 7507575 B2 (BEDINGHAM et al.) 24 March 2009 See the whole document.	1-12, 18-19, 33-39
A	US 2012-0101740 A1 (ORPANA et al.) 26 April 2012 See the whole document.	1-12, 18-19, 33-39

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
"E" earlier application or patent but published on or after the international filing date	"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
"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)	"&" document member of the same patent family
"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	

Date of the actual completion of the international search

16 October 2013 (16.10.2013)

Date of mailing of the international search report

16 October 2013 (16.10.2013)

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 Bcom

Telephone No. +82-42-481-3371



INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2013/053512

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2011-0223602 A1	15/09/2011	AU 2007-333040 A1	19/06/2008
		AU 2007-333040 B2	07/02/2013
		CA 2672034 A1	19/06/2008
		CN 101657548 A	24/02/2010
		CN 101657548 B	10/10/2012
		CN 102851369 A	02/01/2013
		EP 2099930 A2	16/09/2009
		JP 2010-512752 A	30/04/2010
		KR 10-2009-0100380 A	23/09/2009
		US 2008-305481 A1	11/12/2008
		US 2013-116141 A1	09/05/2013
		US 7955802 B2	07/06/2011
		US 8288105 B2	16/10/2012
		WO 2008-074023 A2	19/06/2008
		WO 2008-074023 A3	28/08/2008
US 2010-0324834 A1	23/12/2010	CN 101821609 A	01/09/2010
		EP 2193356 A2	09/06/2010
		US 8374802 B2	12/02/2013
		WO 2009-027102 A2	05/03/2009
		WO 2009-027102 A3	18/06/2009
US 2012-0040352 A1	16/02/2012	AU 2005-295298 A1	27/04/2006
		AU 2005-295298 B2	14/07/2011
		BR PI0517105 A	30/09/2008
		CA 2584569 A1	27/04/2006
		CN 101076607 A	21/11/2007
		CN 101076607 B	24/04/2013
		DE 602005025791 D1	17/02/2011
		DK 1805199T3	26/04/2011
		EP 1805199 A2	11/07/2007
		EP 1805199 B1	05/01/2011
		EP 2351766 A2	03/08/2011
		HK1106251 A1	16/12/2011
		JP 2008-516612 A	22/05/2008
		KR 10-2007-0090159 A	05/09/2007
		KR 10-2013-0021465 A	05/03/2013
		NO 20072506 A	11/07/2007
		PT 1805199 E	11/04/2011
		RU 2007118545 A	27/11/2008
		RU 2460804 C2	10/09/2012
		US 2006-177841 A1	10/08/2006
		US 2013-095479 A1	18/04/2013
		US 7632642 B2	15/12/2009
		WO 2006-044994 A2	27/04/2006
		WO 2006-044994 A3	08/03/2007
US 7507575 B2	24/03/2009	AU 2006-232922 A1	12/10/2006
		AU 2006-232930 A1	12/10/2006

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2013/053512

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2012-0101740 A1	26/04/2012	CA 2602587 A1	12/10/2006
		CN 101156059 B	08/06/2011
		CN 101189505 B	09/11/2011
		DE 602006015043 D1	05/08/2010
		EP 1866631 A1	19/12/2007
		EP 1866631 B1	27/04/2011
		EP 1872112 A1	02/01/2008
		EP 1872112 B1	23/06/2010
		EP 2207026 A1	14/07/2010
		JP 05021614 B2	12/09/2012
		JP 05021615 B2	12/09/2012
		JP 2008-534966 A	28/08/2008
		JP 2008-537594 A	18/09/2008
		KR 10-1265362 B1	20/05/2013
		KR 10-2007-0122525 A	31/12/2007
		KR 10-2008-0014758 A	14/02/2008
		KR 10-2013-0050391 A	15/05/2013
		MX 2007012159 A	10/12/2007
		US 2006-0223169 A1	05/10/2006
		US 2006-0223172 A1	05/10/2006
		US 2009-0218517 A1	03/09/2009
		US 7709249 B2	04/05/2010
		WO 2006-107619 A1	12/10/2006
		WO 2006-107627 A1	12/10/2006
		CN 102576390 A	11/07/2012
		EP 2427843 A1	14/03/2012
		FI 20095514 A0	07/05/2009
		WO 2010-128206 A1	11/11/2010

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: STEINFL, ALESSANDRO STEINFL & BRUNO LLP 155 NORTH LAKE AVENUE, SUITE 700 PASADENA, CALIFORNIA 91101 USA
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PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)

Date of mailing (day/month/year) 16 October 2013 (16.10.2013)

Applicant's or agent's file reference P1272-PCT

FOR FURTHER ACTION

See paragraph 2 below

International application No. PCT/US2013/053512

International filing date (day/month/year) 02 August 2013 (02.08.2013)
--

Priority date(day/month/year) 03 August 2012 (03.08.2012)

International Patent Classification (IPC) or both national classification and IPC

C12Q 1/68(2006.01)i, C12M 1/38(2006.01)i, C12N 15/11(2006.01)i

Applicant

CALIFORNIA INSTITUTE OF TECHNOLOGY

1. This opinion contains indications relating to the following items:

- | | | |
|-------------------------------------|--------------|--|
| ☒ | Box No. I | Basis of the opinion |
| ☐ | Box No. II | Priority |
| ☒ | Box No. III | Non-establishment of opinion with regard to novelty, inventive step and industrial applicability |
| ☐ | Box No. IV | Lack of unity of invention |
| ☒ | Box No. V | Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement |
| ☐ | Box No. VI | Certain documents cited |
| ☒ | Box No. VII | Certain defects in the international application |
| ☐ | Box No. VIII | Certain observations on the international application |

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.
For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/KR Korean Intellectual Property Office 189 Cheongsu-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea Facsimile No. +82-42-472-7140	Date of completion of this opinion 16 October 2013 (16.10.2013)	Authorized officer KIM Seung Beom Telephone No. +82-42-481-3371
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Form PCT/ISA/237 (cover sheet) (July 2011)

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US2013/053512

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of :
 - ☒ the international application in the language in which it was filed
 - ☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))
3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of:
 - a. a sequence listing filed or furnished
 - ☐ on paper
 - ☐ in electronic form
 - b. time of filing or furnishing
 - ☐ contained in the international application as filed.
 - ☐ filed together with the international application in electronic form.
 - ☐ furnished subsequently to this Authority for the purposes of search.
4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
5. Additional comments:

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/US2013/053512

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application

☒ claims Nos. 13-17,20-32

because:

☐ the said international application, or the said claims Nos. _____
relate to the following subject matter which does not require an international search (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 14-17,23-26
are so unclear that no meaningful opinion could be formed (*specify*):

Claims 14-17 and 23-26 are unclear since they are referring to the multiple dependent claims which do not comply with PCT Rule 6.4(a).

☐ the claims, or said claims Nos. _____ are so inadequately supported
by the description that no meaningful opinion could be formed (*specify*):

☒ no international search report has been established for said claims Nos. 13-17,20-32

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.

☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.

☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13ter.1(a) or (b).

☐ See Supplemental Box for further details.

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US2013/053512

**Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability;
citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	1-12,18-19,33-39	YES
	Claims	NONE	NO
Inventive step (IS)	Claims	1-12,18-19,33-39	YES
	Claims	NONE	NO
Industrial applicability (IA)	Claims	1-12,18-19,33-39	YES
	Claims	NONE	NO

2. Citations and explanations :

Reference is made to the following documents:

D1: PLoS One, Vol.7, Issue 1, Article No.e30340, pp.1-8 (January 2012)

D2: US 2011-0223602 A1 (WHITMAN et al.) 15 September 2011

D3: US 2010-0324834 A1 (TREPTOW et al.) 23 December 2010

D4: US 2012-0040352 A1 (WANGH et al.) 16 February 2012

D5: US 7507575 B2 (BEDINGHAM et al.) 24 March 2009

D6: US 2012-0101740 A1 (ORPANA et al.) 26 April 2012

D1 discloses the method permitting the multiplex detection of multiple targets in a single detection channel (See abstract).

1. Novelty and Inventive Step

1.1. Claims 1-12 and 18-19

Claim 1 relates to a method comprising (S1) detecting a plurality of different amplicons generated during a multiplexed amplification reaction, using a single channel detector generating a sum amplitude signal in correspondence of the plurality of different amplicons; (S2) quantifying the plurality of detected different amplicons and (S3) verifying the plurality of detected different amplicons, wherein the quantifying and the verifying comprise using mathematical analysis of the generated sum amplitude signal.

D1, which is considered to be the closest prior art to the subject matter of claim 1, discloses a multiplex detection method, which allows the detection of up to four targets in a single detection channel (See page 1). Also, D1 describes that (S1'-S3') a multiplex PCR is a tool for detection and quantification of specific sequences in a sample and there are at least two probes for two different targets in the multiplex PCR reaction of the system, wherein said

Continued on Supplemental Box

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US2013/053512

Box No. VII Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Claims 13, 20-22 and 27-32 do not comply with the third sentence of PCT Rule 6.4(a), because multiple dependent claims should not serve as a basis for any other multiple dependent claim.

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/US2013/053512

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of : Box No. V

probes are used for amplification and melting curve analysis; and are designed to have a unique T_m which can be recognised in a melting curve analysis (See pages 1 and 3).

Claim 1 differs from D1 in generating the sum amplitude signal used for the analysis of multiplexed reaction. Furthermore, said feature of claim 1 is not obvious to a person skilled in the art by the prior documents, taken alone or in combination. Therefore, claim 1 meets the requirements of PCT Article 33(2) and 33(3) with respect to novelty and inventive step.

Claims 2-12 and 18-19, ultimately dependent on claim 1, also meet the requirements of PCT Article 33(2) and 33(3).

1.2. Claims 33-36

Claim 33 relates to a processor-based hardware analyzer for analyzing a multiplexed amplification reaction, wherein the processor-based hardware analyzer is configured to quantify and verify the multiplexed amplification reaction based on a provided digital representation of a sum amplitude signal in correspondence of a plurality of different amplicons of the multiplexed amplification reaction.

None of the prior art document D1-D6 disclose or suggest the processor-based hardware analyzer providing digital representation of the sum amplitude signal of the present invention. Accordingly, the subject matter of claim 33 is not anticipated nor obvious to a person skilled in the art by the prior documents, taken alone or in combination. Therefore, claim 33 meets the requirements of PCT Article 33(2) and (3) with respect to novelty and inventive step.

Claims 34-36, ultimately dependent on claim 33, also meet the requirements of PCT Article 33(2) and 33(3).

1.3. Claims 37-39

Claim 37 relates to an apparatus for performing a multiplexed amplification reaction, the apparatus comprising a single channel detector configured to detect a sum amplitude signal in correspondence of a plurality of different amplicons of the multiplexed amplification reaction and generate a digital representation of said signal and the processor-based analyzer of any one of claims 33-35, configured to receive the digital representation of the sum amplitude signal.

Continued on The Next Page

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/US2013/053512

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of : Previous Page

None of the prior art document D1-D6 disclose or suggest the apparatus for performing a multiplexed amplification reaction of the present invention. Accordingly, the subject matter of claim 37 is not anticipated nor obvious to a person skilled in the art by the prior documents, taken alone or in combination. Therefore, claim 37 meets the requirements of PCT Article 33(2) and (3) with respect to novelty and inventive step.

Claims 38-39, dependent on claim 37, also meet the requirements of PCT Article 33(2) and 33(3).

2. Industrial Applicability

Claims 1-12, 18-19 and 33-39 are industrially applicable under PCT Article 33(4).

Electronic Acknowledgement Receipt	
EFS ID:	42287417
Application Number:	16864744
International Application Number:	
Confirmation Number:	9929
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil P. Kartalov
Customer Number:	21971
Filer:	Nicholas Shaofeng Tiee/Christina Campbell
Filer Authorized By:	Nicholas Shaofeng Tiee
Attorney Docket Number:	44158-727.304
Receipt Date:	25-MAR-2021
Filing Date:	01-MAY-2020
Time Stamp:	20:02:37
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
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Total Files Size (in bytes):			83904334		
This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503. <u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application. <u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					

Electronic Acknowledgement Receipt	
EFS ID:	42287570
Application Number:	16864744
International Application Number:	
Confirmation Number:	9929
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil P. Kartalov
Customer Number:	21971
Filer:	Nicholas Shaofeng Tiee/Christina Campbell
Filer Authorized By:	Nicholas Shaofeng Tiee
Attorney Docket Number:	44158-727.304
Receipt Date:	25-MAR-2021
Filing Date:	01-MAY-2020
Time Stamp:	20:04:20
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503. <u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application. <u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor:	KARTALOV EMIL; P. et al.	Group Art Unit:	1631
Serial Number:	16/864,744	Examiner:	NA
Filing or 371 (c) Date:	2020-05-01	CONFIRMATION NO:	9929
Title:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS		

FILED ELECTRONICALLY ON: March 25, 2021

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

INFORMATION DISCLOSURE STATEMENT
UNDER 37 CFR § 1.97

Commissioner for Patents:

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

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

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

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

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

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

-- OR --

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

-- OR --

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

-- OR --

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

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

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

-- OR --

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

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

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

-- AND --

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

D. ☐ 37 CFR § 1.97 (e). Statement.

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

-- AND/OR --

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

-- AND/OR --

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

- E. ☐ *Statement Under 37 C.F.R. §1.704(d).* Each item of information contained in the information disclosure statement was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office or is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office that was received by an individual designated in § 1.56(c) not more than thirty (30) days prior to the filing of this information disclosure statement. This statement is made pursuant to the requirements of 37 C.F.R. §1.704(d) to avoid reduction of the period of adjustment of the patent term for Applicant(s) delay.
- F. ☒ *37 CFR §1.98 (a) (2).* The content of the Information Disclosure Statement is as follows:
- ☐ Copies of each of the references listed on the attached Form PTO/SB/08 are enclosed herewith.
- OR --
- ☒ Copies of U.S. Patent Documents (issued patents and patent publications) listed on the attached Form PTO/SB/08 are not enclosed.
- AND/OR --
- ☒ Copies of Foreign Patent Documents and/or Non Patent Literature Documents listed on the attached Form PTO/SB/08 are enclosed in accordance with 37 CFR §1.98 (a)(2).
- AND/OR --
- ☐ Copies of pending unpublished U.S. patent applications are enclosed in accordance with 37 CFR §1.98 (a) (2) (iii).
- G. ☒ *37 CFR §1.98(a)(3).* The Information Disclosure Statement includes non-English patents and/or references.
- ☐ Pursuant to 37 CFR §1.98(a)(3)(i), a concise explanation of the relevance of each patent, publication or other information provided that is not in English is provided herewith.
- ☐ Pursuant to MPEP 609(B), an English language copy of a foreign search report is submitted herewith to satisfy the requirement for a concise explanation where non-English language information is cited in the search report.
- OR --
- ☐ A concise explanation of the relevance of each patent, publication or other information provided that is not in English is as follows: _____
- ☒ Pursuant to 37 CFR §1.98(a) (3) (ii), a copy of a translation, or a portion thereof, of the non-English language reference(s) is provided herewith.
- H. ☐ *37 CFR §1.98(d).* Copies of patents, publications and pending U.S. patent applications, or other information specified in 37 C.F.R. § 1.98(a) are not provided herewith because:
- ☐ Pursuant to 37 CFR §1.98(d)(1) the information was previously submitted in an Information Disclosure Statement, or cited by examiner for another application under which this application claims priority for an earlier effective filing date under 35 U.S.C. 120.
- Application in which the information was submitted: _____
- Information Disclosure Statement(s) filed on: _____
- AND
- ☐ The information disclosure statement submitted in the earlier application complied with paragraphs (a) through (c) of 37 CFR §1.98.

- I. ☒ *Fee Authorization.* The Commissioner is hereby authorized to charge the above-referenced fees of \$0.00 and charge any additional fees or credit any overpayment associated with this communication to Deposit Account No. 23-2415 (Docket No.44158-727.304).

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI

Dated: March 25, 2021

By: /Nicholas Tiee/
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			Application Number	16864744	
			Filing Date	05-01-2020	
			First Named Inventor	KARTALOV EMIL; P.	
			Art Unit	1631	
			Examiner Name	NA	
			Attorney Docket Number	44158-727.304	
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U. S. PATENT DOCUMENTS					
Examiner Initials*	Cite No. ¹	Document Number	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		Number-Kind Code ² (if known)			
	001	US-5079351	01-07-1992	SNINSKY; John J. et al.	
	002	US-5538848	07-23-1996	LIVAK; Kenneth J. et al.	
	003	US-5618711	04-08-1997	GELFAND; David H. et al.	
	004	US-5677152	10-14-1997	BIRCH; David Edward et al.	
	005	US-5723591	03-03-1998	LIVAK; Kenneth J. et al.	
	006	US-5773258	06-30-1998	BIRCH; David Edward et al.	
	007	US-5789224	08-04-1998	GELFAND; David H. [Us] et al.	
	008	US-5804375	09-08-1998	GELFAND; David H. et al.	
	009	US-5834203	11-10-1998	KATZIR; Nir et al.	
	010	US-5876930	03-02-1999	LIVAK; Kenneth J. et al.	
	011	US-5882856	03-16-1999	SHUBER; Anthony P.	
	012	US-5928862	07-27-1999	MORRISON; Larry E.	
	013	US-5948360	09-07-1999	RAO; Prabhakar P. et al.	
	014	US-5981180	11-09-1999	CHANDLER; Van S. et al.	
	015	US-5994056	11-30-1999	HIGUCHI; Russell G.	
	016	US-6030787	02-29-2000	LIVAK; Kenneth J. et al.	
	017	US-6103463	08-15-2000	CHEFTERIN; Alexander B. et al.	
	018	US-6127155	10-03-2000	GELFAND; David H. et al.	
	019	US-6171785	01-09-2001	HIGUCHI; Russell G.	
	020	US-6258569	07-10-2001	LIVAK; Kenneth J. et al.	
	021	US-6534266	03-18-2003	SINGER; Robert H.	

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		Number-Kind Code ² (if known)			
	022	US-6534274	03-18-2003	BECKER; Michael M. et al.	
	023	US-6548259	04-15-2003	WARD; David C. et al.	
	024	US-6642062	11-04-2003	KAUVAR; Lawrence M. et al.	
	025	US-6893875	05-17-2005	TSUJI; Yasuhiro et al.	
	026	US-7101663	09-05-2006	GODFREY; Tony E. et al.	
	027	US-7141377	11-28-2006	GELFAND; David H. et al.	
	028	US-7348141	03-25-2008	FRENCH; David John et al.	
	029	US-7385043	06-10-2008	KRAMER; Fred R.	
	030	US-7410764	08-12-2008	GOCKE; Christopher et al.	
	031	US-7413708	08-19-2008	MAYRAND; Paul E.	
	032	US-7473767	01-06-2009	DIMITROV; Krassen	
	033	US-7507575	03-24-2009	BEDINGHAM; William [Us] et al.	
	034	US-7575864	08-18-2009	BEDZYK; Laura A. et al.	
	035	US-7667024	02-23-2010	MAO; FEI et al.	
	036	US-7671184	03-02-2010	HAENER; Robert et al.	
	037	US-7709249	05-04-2010	BEDINGHAM; William et al.	
	038	US-7767423	08-03-2010	KOPRESKI; Michael [Us] et al.	
	039	US-7771949	08-10-2010	KRAMER; Fred R.	
	040	US-7919237	04-05-2011	DIMITROV; Krassen et al.	
	041	US-7919244	04-05-2011	MADEJON; Seiz Antonio et al.	

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		Number-Kind Code ² (if known)			
	042	US-7930106	04-19-2011	CARRICK; James M. [Us] et al.	
	043	US-7941279	05-10-2011	HWANG; Jenq-Neng et al.	
	044	US-8039215	10-18-2011	HIGUCHI; Russell [Us] et al.	
	045	US-8148512	04-03-2012	DIMITROV; Krassen et al.	
	046	US-8426132	04-23-2013	LI; BIN [Us] et al.	
	047	US-8455184	06-04-2013	ATCHLEY; Daniel H. [Us] et al.	
	048	US-8492094	07-23-2013	DIMITROV; Krassen [Us] et al.	
	049	US-8519115	08-27-2013	WEBSTER; Philippa J. [Us] et al.	
	050	US-8614061	12-24-2013	BRABETZ; Werner [De] et al.	
	051	US-8771955	07-08-2014	REED; Michael W. [Us] et al.	
	052	US-8838394	09-16-2014	KARTALOV; Emil [Us] et al.	
	053	US-8877464	11-04-2014	BABIEL; Reiner [De] et al.	
	054	US-8962250	02-24-2015	STANLEY; Keith [Au] et al.	
	055	US-9133506	09-15-2015	KATZIR; NIR [Il] et al.	
	056	US-9222128	12-29-2015	SAXONOV; Serge et al.	
	057	US-9260761	02-16-2016	TYAGI; Sanjay [Us] et al.	
	058	US-9366632	06-14-2016	LINK; Darren R. et al.	
	059	US-9422593	08-23-2016	ROTHMANN; Thomas [De] et al.	
	060	US-9441266	09-13-2016	LARSON; Jonathan William et al.	

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		Number-Kind Code ² (if known)			
	061	US-9447457	09-20-2016	CHUN; Jong Yoon [Kr] et al.	
	062	US-9458497	10-04-2016	HASSIBI; Arjang et al.	
	063	US-9791372	10-17-2017	MALIK; Imran R. et al.	
	064	US-10066263	09-04-2018	RAJAGOPAL; Aditya et al.	
	065	US-10068051	09-04-2018	KARTALOV; Emil et al.	
	066	US-10068051	09-04-2018	KARTALOV; Emil et al.	
	067	US-10301668	05-28-2019	JACKY; Lucian A.E.	
	068	US-10770170	09-08-2020	KARTALOV; Emil P. et al.	
	069	US-20020022273	02-21-2002	EMPEDOCLES; Stephen A. [Us] et al.	
	070	US-20020146734	10-10-2002	ORTYN; William E. et al.	
	071	US-20030134320	07-17-2003	BARRUS; Jeffrey K. et al.	
	072	US-20030148280	08-07-2003	HARRIS; Barbara J. et al.	
	073	US-20030148544	08-07-2003	NIE; Shuming et al.	
	074	US-20040053230	03-18-2004	SCHAFFER; Michael Edward [Us] et al.	
	075	US-20040191794	09-30-2004	WEINDEL; Kurt et al.	
	076	US-20050053950	03-10-2005	ZUDAIRE UBANI; Enrique et al.	
	077	US-20050064435	03-24-2005	SU; Xing et al.	
	078	US-20050106607	05-19-2005	YIN; Li-Te et al.	
	079	US-20050164264	07-28-2005	SHIPWASH; Edward	
	080	US-20050214753	09-29-2005	SHULTZ; John W. et al.	

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		Number-Kind Code ² (if known)			
	081	US-20050250146	11-10-2005	MCMILLAN; William A.	
	082	US-20050260640	11-24-2005	ANDERSEN; Mark R. et al.	
	083	US-20060216708	09-28-2006	VENEMA; Fokke	
	084	US-20070072211	03-29-2007	NEWTON; Nick et al.	
	085	US-20070178485	08-02-2007	EL-DEIRY; Wafik S. et al.	
	086	US-20070231824	10-04-2007	CHEE; Mark S. et al.	
	087	US-20080050737	02-28-2008	ARIELI; Boaz et al.	
	088	US-20080069733	03-20-2008	MALTEZOS; George et al.	
	089	US-20080096767	04-24-2008	KOHN; Heinz G.	
	090	US-20080124705	05-29-2008	KRAMER; Fred R.	
	091	US-20090042735	02-12-2009	BLAIR; Steven M. et al.	
	092	US-20090048785	02-19-2009	KATZIR; NIR et al.	
	093	US-20090062129	03-05-2009	MCKERNAN; Kevin [Us] et al.	
	094	US-20100015607	01-21-2010	GEISS; Gary K. et al.	
	095	US-20100041092	02-18-2010	LIN; Michael Z. et al.	
	096	US-20100047924	02-25-2010	WEBSTER; Philippa J. et al.	
	097	US-20100112710	05-06-2010	GEISS; Gary K. et al.	
	098	US-20100120043	05-13-2010	SOOD; Anup et al.	
	099	US-20100151443	06-17-2010	XIANG; Qing et al.	
	100	US-20100159447	06-24-2010	LI; BIN et al.	
	101	US-20100210472	08-19-2010	EMPEDOCLES; Stephen A. et al.	

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		Number-Kind Code ² (if known)			
	102	US-20100233686	09-16-2010	HIGUCHI; Russell et al.	
	103	US-20100248257	09-30-2010	JACOBSEN; Kivin et al.	
	104	US-20100261026	10-14-2010	FERREE; Sean M. et al.	
	105	US-20100267064	10-21-2010	KARTALOV; Emil P. et al.	
	106	US-20100273173	10-28-2010	HIRAI; Mitsuharu et al.	
	107	US-20100324834	12-23-2010	TREPTOW; Rainer [De] et al.	
	108	US-20110104684	05-05-2011	HOOPER; Dennis G.	
	109	US-20110151459	06-23-2011	ROTHMANN; Thomas et al.	
	110	US-20110151550	06-23-2011	SAGNER; Gregor et al.	
	111	US-20110171658	07-14-2011	CARRICK; James M.	
	112	US-20110207623	08-25-2011	DIMITROV; Krassen et al.	
	113	US-20110223602	09-15-2011	WHITMAN; Douglas F. [Us] et al.	
	114	US-20110237459	09-29-2011	NOVA; Michael P. et al.	
	115	US-20120003646	01-05-2012	JOO; Sunghae A. et al.	
	116	US-20120040349	02-16-2012	VON; Lode Piia et al.	
	117	US-20120040352	02-16-2012	WANGH; Lawrence J. [Us] et al.	
	118	US-20120045756	02-23-2012	ROTHMANN; Thomas et al.	
	119	US-20120077195	03-29-2012	LI; Qingge et al.	
	120	US-20120101740	04-26-2012	ORPANA; Arto [Se] et al.	
	121	US-20120122704	05-17-2012	ATCHLEY; Daniel H. et al.	

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			Application Number	16864744	
			Filing Date	05-01-2020	
			First Named Inventor	KARTALOV EMIL; P.	
			Art Unit	1631	
			Examiner Name	NA	
			Attorney Docket Number	44158-727.304	
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U. S. PATENT DOCUMENTS					
Examiner Initials*	Cite No. ¹	Document Number	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		Number-Kind Code ² (if known)			
	122	US-20120141995	06-07-2012	LI; Qingge et al.	
	123	US-20120171677	07-05-2012	LUDOWISE; Peter D.	
	124	US-20120184017	07-19-2012	CHATTERJEE; DEB K.	
	125	US-20120190030	07-26-2012	CHUN; Jong Yoon et al.	
	126	US-20120196283	08-02-2012	BABIEL; Reiner et al.	
	127	US-20120244534	09-27-2012	CHING; Jesus et al.	
	128	US-20120252014	10-04-2012	LOEFFERT; Dirk et al.	
	129	US-20120252017	10-04-2012	REED; Michael W. et al.	
	130	US-20120258457	10-11-2012	JAROSCH; Florian et al.	
	131	US-20120302448	11-29-2012	HUTCHISON; John Brian et al.	
	132	US-20130017971	01-17-2013	GEISS; Gary K. [Us] et al.	
	133	US-20130040841	02-14-2013	SAXONOV; Serge et al.	
	134	US-20130078626	03-28-2013	WASSERSTROM; Adam et al.	
	135	US-20130116780	05-09-2013	MILLER; Eran et al.	
	136	US-20130261019	10-03-2013	LIN; Chenxiang [Us] et al.	
	137	US-20130288244	10-31-2013	DECIU; Cosmin et al.	
	138	US-20140004520	01-02-2014	MOHAPATRA; Shyam S. et al.	
	139	US-20140038195	02-06-2014	MALIK; Imran R. [Us] et al.	
	140	US-20140171341	06-19-2014	JOUVENOT; Yann et al.	
	141	US-20140213471	07-31-2014	RAJAGOPAL; Aditya et al.	

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	142	US-20140274774	09-18-2014	LI; Kelly et al.	
	143	US-20150057178	02-26-2015	KARTALOV; Emil et al.	
	144	US-20150140554	05-21-2015	SNYDER; Richard O. et al.	
	145	US-20150211054	07-30-2015	KOSTEM; Emrah et al.	
	146	US-20150275295	10-01-2015	WANG; Linfa et al.	
	147	US-20160040256	02-11-2016	CHEN; Caifu et al.	
	148	US-20160108464	04-21-2016	SAXONOV; Serge et al.	
	149	US-20160201122	07-14-2016	BUSHKIN; Yuri et al.	
	150	US-20160273048	09-22-2016	ROPERCH; Jean-Pierre	
	151	US-20170145490	05-25-2017	CHIU; Daniel T. et al.	
	152	US-20170314073	11-02-2017	GRÖMMINGER; Sebastian et al.	
	153	US-20170362636	12-21-2017	RAJAGOPAL; Aditya et al.	
	154	US-20180030551	02-01-2018	RAJAGOPAL; Aditya et al.	
	155	US-20180052110	02-22-2018	MALIK; Imran R. et al.	
	156	US-20180057864	03-01-2018	JACKY; Lucien A. E. et al.	
	157	US-20190002963	01-03-2019	RAJAGOPAL; Aditya	
	158	US-20190032112- A1	01-31-2019	RAJAGOPAL; Aditya et al.	
	159	US-20190112636	04-18-2019	RAJAGOPAL; Aditya et al.	
	160	US-20190233882	08-01-2019	JACKY; Lucien et al.	
	161	US-20200010876- A1	01-09-2020	MACDONALD; Christopher	

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		Country Code ³ Number ⁴ Kind Code ⁵ (if known)				
	001	CN-101570782-A	11-04-2009	CHUNHUA YANG [CN]	Abstract:	☒
	002	CN-1936019-A	03-28-2007	UNIV XIAMEN [CN]	Abstract:	☒
	003	EP-0519338-A1	12-23-1992	HOFFMANN LA ROCHE [CH]		☐
	004	EP-1448581-B1	11-12-2008	INST SYSTEMS BIOLOGY [US]		☐
	005	EP-1629108-B1	12-31-2014	PHRI PROPERTIES INC [US]		☐
	006	EP-1963531-B1	09-21-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	007	EP-2224017-A1	09-01-2010	ARKRAY INC [JP]		☐
	008	WO-0107640-A2	02-01-2001	SEC DEP HOME DEPT [GB], et al.		☐
	009	WO-02056014-A2	07-18-2002	GLAXO GROUP LTD [GB], et al.		☐
	010	WO-02056014-A3	10-09-2003	GLAXO GROUP LTD [GB], et al.		☐

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		Country Code ³ Number ⁴ Kind Code ⁵ (if known)				
	011	WO-03002979-A2	01-09-2003	ILLUMINA INC [US], et al.		☐
	012	WO-03020967-A1	03-13-2003	ROCHE DIAGNOSTICS GMBH [DE], et al.		☐
	013	WO-2004087950-A2	10-14-2004	ROCHE DIAGNOSTICS GMBH [DE], et al.		☐
	014	WO-2006079049-A2	07-27-2006	THIRD WAVE TECH INC [US], et al.		☐
	015	WO-2007076128-A2	07-05-2007	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	016	WO-2007076128-A3	11-29-2007	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	017	WO-2007076129-A2	07-05-2007	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	018	WO-2007076129-A3	03-06-2008	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	019	WO-2007076132-A2	07-05-2007	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	020	WO-2007076132-A3	09-07-2007	NANOSTRING TECHNOLOGIES INC [US], et al.		☐

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		Country Code ³ Number ⁴ Kind Code ⁵ (if known)				
	021	WO-2007139766-A2	12-06-2007	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	022	WO-2007139766-A3	12-11-2008	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	023	WO-2008118998-A2	10-02-2008	PRIMERA BIOSYSTEMS INC [US], et al.		☐
	024	WO-2008124847-A2	10-16-2008	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	025	WO-2008124847-A3	02-19-2009	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	026	WO-2009036514-A2	03-26-2009	MOLECULAR PLANT BREEDING NOMINEES LTD [AU], et al.		☐
	027	WO-2010007355-A1	01-21-2010	LUMORA LTD [GB], et al.		☐
	028	WO-2010013017-A1	02-04-2010	OXITEC LTD [GB], et al.		☐
	029	WO-2010017543-A1	02-11-2010	SMITHS DETECTION INC [US], et al.		☐
	030	WO-2010019826-A1	02-18-2010	NANOSTRING TECHNOLOGIES INC [US], et al.		☐

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	031	WO-2010128206-A1	11-11-2010	EXPRESSION ANALYTICS OY [FI], et al.		☐
	032	WO-2011047087-A2	04-21-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	033	WO-2011047087-A3	08-18-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	034	WO-2011100541-A2	08-18-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	035	WO-2011100541-A3	01-05-2012	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	036	WO-2011116088-A2	09-22-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	037	WO-2011116088-A3	02-02-2012	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	038	WO-2012058638-A2	05-03-2012	HARVARD COLLEGE [US], et al.		☐
	039	WO-2012058638-A3	12-20-2012	HARVARD COLLEGE [US], et al.		☐
	040	WO-2012106428-A2	08-09-2012	UNIV FLORIDA [US], et al.		☐

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	041	WO-2012135340-A2	10-04-2012	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	042	WO-2012135340-A3	12-20-2012	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	043	WO-2013096851-A1	06-27-2013	HARVARD COLLEGE [US]		☐
	044	WO-2013116780-A1	08-08-2013	CALIFORNIA INST OF TECHN [US], et al.		☐
	045	WO-2014022827-A1	02-06-2014	CALIFORNIA INST OF TECHN [US]		☐
	046	WO-2014116884-A1	07-31-2014	CALIFORNIA INST OF TECHN [US]		☐
	047	WO-2015147370-A1	10-01-2015	SEEGENE INC [KR]		☐
	048	WO-2016154600-A1	09-29-2016	MASSACHUSETT S GEN HOSPITAL [US]		☐
	049	WO-2017139354-A1	08-17-2017	UNIV MICHIGAN REGENTS [US]		☐
	050	WO-2017173035-A1	10-05-2017	CHROMACODE INC [US]		☐

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			Application Number	16864744	
			Filing Date	05-01-2020	
			First Named Inventor	KARTALOV EMIL; P.	
			Art Unit	1631	
			Examiner Name	NA	
			Attorney Docket Number	44158-727.304	
Sheet	14	of	27		

FOREIGN PATENT DOCUMENTS						
Examiner Initials*	Cite No. ¹	Foreign Patent Document	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
		Country Code ³ Number ⁴ Kind Code ⁵ (if known)				
	051	WO-2017218777-A1	12-21-2017	CALIFORNIA INST OF TECHN [US]		☐
	052	WO-2018081178-A1	05-03-2018	TWO PORE GUYS INC [US]		☐
	053	WO-2019006023-A1	01-03-2019	CHROMACODE INC [US]		☐
	054	WO-2019079204-A1	04-25-2019	CHROMACODE INC [US]		☐
	055	WO-2019169043-A1	09-06-2019	CHROMACODE INC [US]		☐
	056	WO-9746714-A1	12-11-1997	UNIV UTAH RES FOUND [US], et al.		☐
	057	WO-99/52708	10-21-1999	LUMINEX CORP [US]		☐
	058	WO-9919515-A1	04-22-1999	LUMINEX CORP [US]		☐

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	001	ARYA, et al., Basic principles of real-time quantitative PCR, 2005, Expert Rev. Mol. Diagn., 5(2), pg. 209-219.	☐
	002	BEIGE, et al. Clinical evaluation of a Mycobacterium tuberculosis PCR assay. J Clin Microbiol. 1995 Jan;33(1):90-5.	☐
	003	BLACKET et al. Universal primers for fluorescent labeling of PCR fragments - an efficient and cost-effective approach to genotyping by fluorescence. Molecular Ecology Resources 12(3):456-463 (2012) Epub 2012 Jan 24.	☐
	004	CHAMBERLAIN, et al. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res. 1988 Dec 9;16(23):11141-56.	☐
	005	CHAPIN et al., Rapid microRNA profiling on encoded gel microparticles. Angewandte Chemie International Edition, 50(10):2289-2293, 2011.	☐
	006	CHEN, et al. A Homogeneous, ligase-mediated DNA diagnostic test. Genome Research, 1998, volume 8, pages 549-556.	☐
	007	CHONG, et al. Single-tube multiplex-PCR screen for common deletional determinants of alpha-thalassemia. Blood. 2000 Jan 1;95(1):360-2.	☐
	008	Chromatogram, 2011, 2 pages. Dorland's illustrated medical dictionary. Retrieved online on 22 January 2014 from «http://www.credoreference.com».	☐
	009	CHUN et al. Dual priming oligonucleotide system for the multiplex detection of respiratory viruses and SNP genotyping of CYP2C19 gene. Nucleic Acids Res. 35(6):e40 (2007).	☐

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	010	CRAIG, et al. Ordering of cosmid clones covering Herpes simplex virus type I (HSV-I) genome: a test case for fingerprinting by hybridisation. Nucleic Acids Research, 1990, volume 18, pages 2653-2660.	☐
	011	DOS SANTOS, et al. A simple one-step real-time RT-PCR for diagnosis of dengue virus infection. J Med Virol. 2008 Aug;80(8):1426-33. .	☐
	012	EL-HAJJ, et al. Detection of rifampin resistance in Mycobacterium tuberculosis in a single tube with molecular beacons. J Clin Microbiol. 2001 Nov;39(11):4131-7.	☐
	013	EMBL-Bank: AJ303204. http://www.ebi.ac.uk/Tools/dbfetch/emblfetch?db=embl&id=AJ303204&format=default&style=default&Retrieve=Retrieve . Accessed February 2012.	☐
	014	EMBL-Bank: GQ395623. http://www.ebi.ac.uk/Tools/dbfetch/emblfetch?db=embl&id=GQ395623&format=default&style=default&Retrieve=Retrieve . Accessed February 2012.	☐
	015	EP17814096.8 Extended European Search Report dated December 2, 2019.	☐
	016	European Office Action for Patent Application No. EP13824744.0 dated November 6, 2018.	☐
	017	European Patent Application No. 17776626 Office Action dated October 18, 2019.	☐
	018	EVANS et al. Digital PCR for Noninvasive Detection of Aneuploidy: Power Analysis Equations for Feasibility. Fetal Diagn. Ther. 31:244-247(2012).	☐
	019	Extended European Search Report for Patent Application No. 13744261.2 dated May 3, 2016.	☐

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	020	Extended European Search Report for Patent Application No. EP13824744.0 dated February 26, 2016.	☐	
	021	FODOR, et al. Multiplexed biochemical assays with biological chips. Nature. 1993 Aug 5;364(6437):555-6.	☐	
	022	FORTINA, et al. Digital mRNA profiling. Nat Biotechnol. 2008 Mar;26(3):293-4.	☐	
	023	FU et al. Multiplex detection and SNP genotyping in a single fluorescence channel. PLoS One 7(1):Article No. e30340 (January 2012).	☐	
	024	GANDELMAN, et al., Novel Bioluminescent Quantitative Detection of Nucleic Acid Amplification in Real-Time, PLoS One, 2010, 5(11):e14155, 14 pages.	☐	
	025	GenBank: M93130.1. Dengue type 3 virus complete genome RNA, complete cds. http://www.ncbi.nlm.nih.gov/nuccore/M93130 . Accessed February 2012.	☐	
	026	HAN, et al., Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. Nature Biotechnology 19.99 (July 2001): 631-635.	☐	
	027	HARTMAN, et al. Development of a novel internal positive control for Taqman based assays. Mol Cell Probes. 2005 Feb;19(1):51-9. Epub 2004 Dec 10.	☐	
	028	HAUSTEIN et al. Fluorescence correlation spectroscopy: novel variations of an established technique. Ann Rev Biophys Biomol Struct 36:151-69 (2007).	☐	
	029	HEIDARI, et al. Detection of Plasmodium falciparum Directly from Blood Samples Using the Polymerase Chain Reaction. Journal of Sciences, Islamic Republic of Iran. 2005 16(1):21-24.	☐	

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	030	HENEGARIU, et al. Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques. 1997 Sep;23(3):504-11.		☐
	031	HIV databases. http://www.hiv.lanl.gov/content/index . Accessed February 2012.		☐
	032	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.		☐
	033	HOREJSH, et al. A molecular beacon, bead-based assay for the detection of nucleic acids by flow cytometry. Nucleic Acids Res. 2005 Jan 19;33(2):e13.		☐
	034	HUANG, et al. Identification of 8 foodborne pathogens by multicolor combinational probe coding technology in a single real-time PCR. Clin Chem. 2007 Oct;53(10):1741-8. Epub 2007 Aug 10.		☐
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	037	International Search Report and Written Opinion dated 11/21/19 for PCT/US2019/050050.		☐
	038	JOTHIKUMAR, et al. Design of FRET-TaqMan probes for multiplex real-time PCR using an internal positive control. Biotechniques. 2009 Jun;46(7):519-24. .		☐
	039	KLOSTRANEC et al., Convergence of quantum dot barcodes with microfluidics and signal processing for multiplexed high-throughput infectious disease diagnostics. Nano Letters, 7(9):2812-2818, 2007.		☐

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	040	KUHN et al. Hybridization of DNA and PNA molecular beacons to single-stranded and double-stranded DNA targets. J Am Chem Soc. 124(6):1097-103 (2002).		☐
	041	LAO, et al. Multiplexing RT-PCR for the detection of multiple miRNA species in small samples. Biochem Biophys Res Commun. 2006 Apr 28;343(1):85-9. Epub 2006 Feb 28.		☐
	042	LEE, et al. Novel multiplex PCR using dual-priming oligonucleotides for detection and discrimination of the Mycobacterium tuberculosis complex and M. bovis BCG. J Clin Microbiol. 2010 Dec;48(12):4612-4. Epub 2010 Oct 13.		☐
	043	LEE, et al. Seven-color, homogeneous detection of six PCR products. Biotechniques. 1999 Aug;27(2):342-9.		☐
	044	LI et al., Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes. Nature Biotechnology, 23(7):885-889, 2005.		☐
	045	LIEW, et al. Validating a custom multiplex ELISA against individual commercial immunoassays using clinical samples. Biotechniques. 2007 Mar;42(3):327-8, 330-3.		☐
	046	LIN et al., Self-assembled combinatorial encoding nanoarrays for multiplexed biosensing. Nano Letters, 7(2):507-512, 2007.		☐
	047	LIVAK, et al. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. 1995 Jun;4(6):357-62.		☐
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	049	LOGAN,et al., An Overview of real-time PCR platforms. 2004, In Real-time PCR: An essential guide, pg. 13-30.		☐

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	051	MORRISON, et al. Two-color ratio-coding of chromosome targets in fluorescence in situ hybridization: quantitative analysis and reproducibility. Cytometry. 1997 Apr 1;27(4):314-26.	☐	
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	054	NOORDHOEK, et al. Sensitivity and specificity of PCR for detection of Mycobacterium tuberculosis: a blind comparison study among seven laboratories. J Clin Microbiol. 1994 Feb;32(2):277-84.	☐	
	055	OLIVEIRA, et al. Multiplex PCR strategy for rapid identification of structural types and variants of the mec element in methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother. 2002 Jul;46(7):2155-61.	☐	
	056	OU, et al. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science. 1988 Jan 15;239(4837):295-7.	☐	
	057	PATON, et al. Detection and characterization of Shiga toxigenic Escherichia coli by using multiplex PCR assays for stx1, stx2, eaeA, enterohemorrhagic E. coli hlyA, rfbO111, and rfbO157. J Clin Microbiol. 1998 Feb;36(2):598-602.	☐	
	058	PATTERSON, et al. Detection of HIV-1 DNA and messenger RNA in individual cells by PCR-driven in situ hybridization and flow cytometry. Science. 1993 May 14;260(5110):976-9.	☐	

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	059	PCT/US19/40392 International Search Report and Written Opinion dated November 06, 2019.	☐
	060	PCT/US2013/024509 International search report and written opinion dated April 12, 2013 .	☐
	061	PCT/US2013/053512 International Search Report and Written Opinion dated October 16, 2013.	☐
	062	PCT/US2017/24933 International search report and written report dated June 22, 2017.	☐
	063	PCT/US2017/37682 International Search Report and Written Opinion dated November 16, 2017.	☐
	064	PCT/US2018/039846 International Preliminary Report on Patentability dated December 31, 2019.	☐
	065	PCT/US2018/055927 International Preliminary Report on Patentability dated April 21, 2020.	☐
	066	PCT/US2018/39846 International Search Report and Written Opinion Mailed September 14, 2018.	☐
	067	PCT/US2019/019906 International Preliminary Report on Patentability September 01, 2020.	☐
	068	PCT/US2019/019906 International Search Report and Written Opinion dated May 06, 2019.	☐
	069	PCT/US2019/027751 International Preliminary Report on Patentability dated October 20, 2020.	☐
	070	PCT/US2019/027751 International Search Report and Written Opinion dated June 26, 2019.	☐
	071	PCT/US2019/035129 International Search Report and Written Opinion dated November 01, 2019.	☐

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			Art Unit	1631	
			Examiner Name	NA	
			Attorney Docket Number	44158-727.304	
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	072	PCT/US2019/035129 Invitation to Pay Additional Fees dated August 19, 2019.		☐
	073	PCT/US2019/040392 International Preliminary Report on Patentability dated January 05, 2021.		☐
	074	PCT/US2019/041239 International Preliminary Report on Patentability dated January 21, 2021.		☐
	075	PCT/US2019/041239 International Search Report and Written Opinion dated September 30, 2019.		☐
	076	PETERSEN, et al. Short PNA molecular beacons for real-time PCR allelic discrimination of single nucleotide polymorphisms. Mol Cell Probes. 2004 Apr;18(2):117-22.		☐
	077	PIERCE, et al. Linear-After-The-Exponential (LATE)-PCR: primer design criteria for high yields of specific single-stranded DNA and improved real-time detection. Proc Natl Acad Sci U S A. 2005 Jun 14;102(24):8609-14. Epub 2005 Jun 3.		☐
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	080	RAJAGOPAL et al. Supercolor Coding Methods for Large-Scale Multiplexing of Biochemical Assays. Analytical Chemistry 85:7629-7636 (2013).		☐
	081	RAJAGOPAL et al., Significant Expansion of Real-Time PCR Multiplexing with Traditional Chemistries using Amplitude Modulation. Scientific Reports. 9(1): 1053 (2019).		☐

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	082	Rickert et al., "Multiplexed Real-Time PCR Using Universal Reporters," Clin. Chem., 50(9):1680- 1683, 2004.		☐
	083	ROSENSTRAUS, et al. An internal control for routine diagnostic PCR: design, properties, and effect on clinical performance. J Clin Microbiol. 1998 Jan;36(1):191-7.		☐
	084	ROTH, et al. Feasibility and efficacy of routine PCR screening of blood donations for hepatitis C virus, hepatitis B virus, and HIV-1 in a blood-bank setting. Lancet. 1999 Jan 30;353(9150):359-63.		☐
	085	SAIKI, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, 239(4839):487-491 (January 29, 1988).		☐
	086	SAMBROOK, et al. Molecular Cloning: A Laboratory Manual. 2nd Edition, 1989.		☐
	087	SANGER et al. DNA sequencing with chain-terminating inhibitors. PNAS USA 1977 Dec;74(12):5463-7 (1977).		☐
	088	Second Office Action for Chinese Patent Application No. 201380049844.9 dated September 19, 2016 (w/translation).		☒
	089	SPEICHER, et al. Karyotyping human chromosomes by combinatorial multi-fluor FISH. Nat Genet. 1996 Apr;12(4):368-75.		☐
	090	ThermoFisher Scientific Fluorescence SpectraViewer. Retrieved from http://www.thermofisher.com/us/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html on September 25, 2015.		☐
	091	TIRASOPHON, et al. A novel detection of a single Plasmodium falciparum in infected blood. Biochem Biophys Res Commun. 1991 Feb 28;175(1):179-84.		☐

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	092	TYAGI, et al. Multicolor molecular beacons for allele discrimination. Nat Biotechnol. 1998 Jan;16(1):49-53.	☐
	093	TYAGI, et al. Wavelength-shifting molecular beacons. Nat Biotechnol. 2000 Nov;18(11):1191-6.	☐
	094	United States Application No. 16/128,343 Final Office Action dated March 16, 2021.	☐
	095	URDEA, et al. Requirements for high impact diagnostics in the developing world. Nature. 2006 Nov 23;444 Suppl 1:73-9.	☐
	096	U.S. Application No. 13/756,760 Notice of allowance dated June 2, 2014.	☐
	097	U.S. Application No. 13/756,760 Office action dated January 24, 2014 .	☐
	098	U.S. Application No. 13/958,479 Office Action dated March 1, 2016.	☐
	099	U.S. Application No. 14/451,876 Office action dated June 6, 2017.	☐
	100	U.S. Application No. 14/451,876 Office action dated November 23, 2016.	☐
	101	U.S. Application No. 15/623,974 Notice of Allowance dated July 3, 2018.	☐
	102	U.S. Application No. 15/623,974 Office Action dated February 22, 2018.	☐
	103	U.S. Application No. 15/677,772 Final Office Action Mailed June 7, 2018.	☐
	104	U.S. Application No. 15/677,772 Non-Final Office Action Mailed February 8, 2018.	☐
	105	U.S. Application No. 15/892,245 First Action Interview - Office Action mailed July 19, 2018.	☐

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	106	U.S. Application No. 15/892,245 Office Action dated December 31, 2018.	☐
	107	U.S. Application No. 15/892,245 Preinterview First Interview Office Action Mailed May 11, 2018.	☐
	108	U.S. Application No. 16/020,673 Non-Final Office Action dated November 27, 2020.	☐
	109	U.S. Application No. 14/451,876 Notice of Allowance dated July 11, 2018.	☐
	110	U.S. Application No. 14/451,876 Notice of Allowance dated June 14, 2018.	☐
	111	U.S. Patent Application No. 15/892,245 Office Action dated December 11, 2019.	☐
	112	U.S. Patent Application No. 15/892,245 Office Action dated June 17, 2019.	☐
	113	U.S. Patent Application No. 16/128,343 Non-Final Office Action dated June 16, 2020.	☐
	114	U.S. Patent Application No. 16/128,343 Office Action dated October 15, 2019.	☐
	115	U.S. Serial No. 15/701,014 Office Action dated January 24, 2020.	☐
	116	U.S. Serial No. 15/914,356 Office Action dated July 22, 2019.	☐
	117	U.S. Serial No. 15/914,356 Office Action dated March 8, 2019.	☐
	118	U.S. Serial No. 16/051,736 Office Action dated August 21, 2020.	☐
	119	U.S. Serial No. 16/128,343 First Action Interview dated April 18, 2019.	☐

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	120	VET, et al. Multiplex detection of four pathogenic retroviruses using molecular beacons. Proc Natl Acad Sci U S A. 1999 May 25;96(11):6394-9.	☐	
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	128	ZHANG, et al. A novel real-time quantitative PCR method using attached universal template probe. Nucleic Acids Res. 2003 Oct 15;31(20):e123(pp. 1-8).	☐	

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	130	ZHAO et al. Comprehensive Algorithm for Quantitative Real-Time Polymerase Chain Reaction. Journal of Computational Biology 12(8):1047-1064 (2005).	☐

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**Espacenet****Bibliographic data: CN101570782 (A) — 2009-11-04**

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

Inventor(s): CHUNHUA YANG [CN] ± (YANG CHUNHUA, ; CAO JIJUAN, ; WANG SHUO)

Applicant(s): CHUNHUA YANG [CN] ± (YANG CHUNHUA, ; XU JUNYI, ; CAO JIJUAN, ; WANG SHUO)

Classification: - **international:** **C12Q1/68; G01N30/02; C12R1/01; C12R1/22; C12R1/37; C12R1/385; C12R1/425**
- **cooperative:**

Application number: CN2009110803 20090320

Priority number(s): CN2009110803 20090320

Also published as: CN101570782 (B)

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/μL and contain specific primer pairs of respective 4 species of target bacteria in an amount of 10 μM each.; The detection kit and the detection method of the invention can detect the 8 species of pathogenic bacteria in the dairy 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.

[19] 中华人民共和国国家知识产权局



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C12R 1/425 (2006.01)
C12R 1/22 (2006.01)
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C12R 1/385 (2006.01)

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[51] Int. Cl. (续)

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代理人 刘晓琴

权利要求书 2 页 说明书 17 页 附图 2 页

[54] 发明名称

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 MgCl₂、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 10 μM。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。

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 MgCl₂、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 MgCl₂、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 CGCTGGCCCCATTCGCTCCAGCGCT
普通变形杆菌	GATGGCAAGTACAAGTAAG GACGCTGAGATTGACCTA
奇异变形杆菌	CAACGTGAGATTAGTGGTGA CTGCTTTAAGTTCACAAATTAAGTG
肺炎克雷伯氏菌	TGGCCCGCGCCCAGGGTTTCGAAA 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，

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°C, 3 min;

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

终止延伸: 72°C, 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°C;

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。

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

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 MgCl₂、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 MgCl₂、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	TGGCCCGCGCCCGGGTTCGAAA 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；

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 检测图谱；

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 CTGCTTIAAGTTCACAAATTAAGTG	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 TTTCAACCGGTAACAGGATTG	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 MgCl₂、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 MgCl₂、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

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

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
------	-----

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

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

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

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 μ L 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

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

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显示为阴性。

SEQUENCE LISTING

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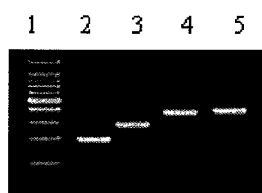


图 1



图 2

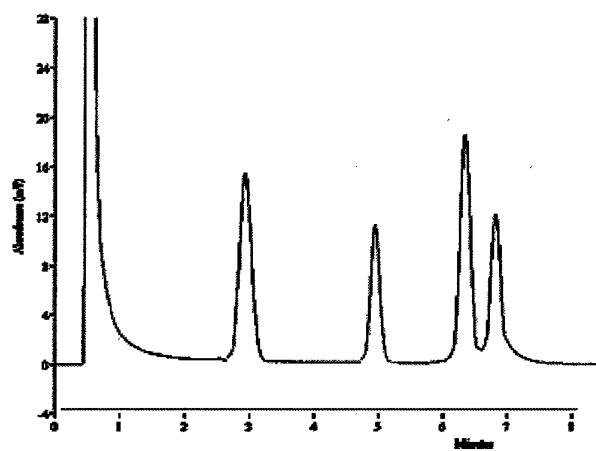


图 3

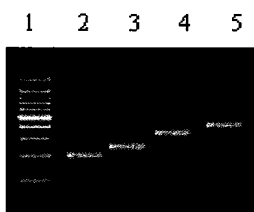


图 4

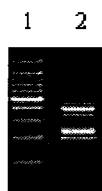


图 5

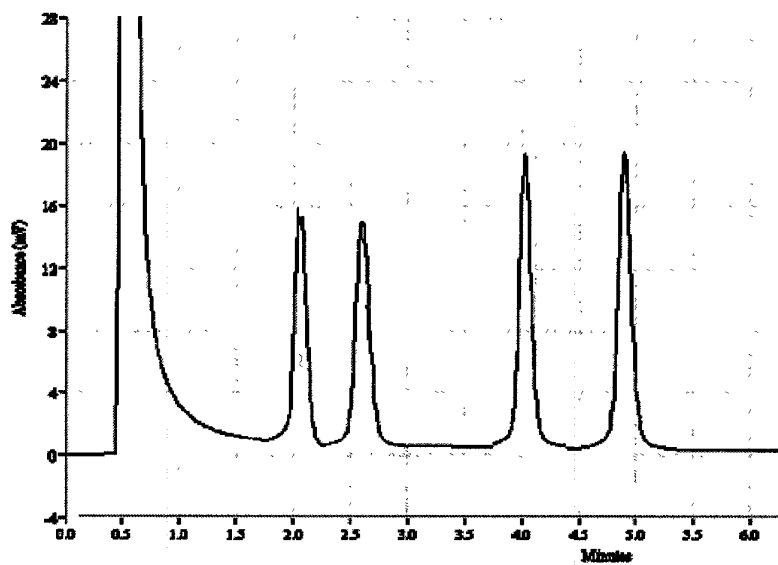


图 6

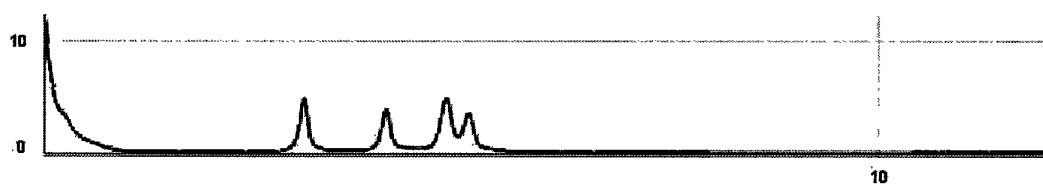


图 7

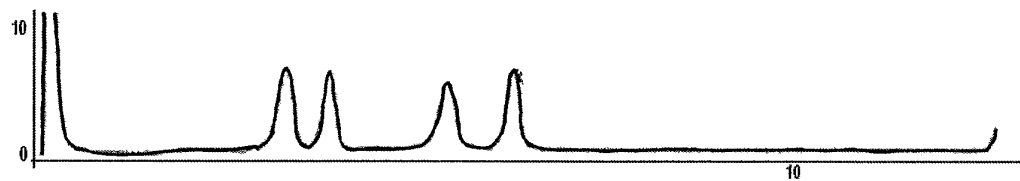


图 8

Bibliographic data

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Abstract of CN1936019A

The invention relates to a probe coding method for multiple real time nucleic acid amplification testing that includes the following steps: using the fluorescent dye of nucleic acid amplification testing instrument as the basal element of fluorescent probe mark that is called fluorescent basal element; marking the specificity probe of target sequence between the fluorescent basal element inter-compounding. Using the quantity of fluorescent basal element, cycling threshold value and the relative intension of the basal elements, the random plural gene specificity fluorescent fingerprint atlas would be gained to testing plural different nucleic acid sequence synchronously under the situation of limited fluorescent basal element quantity. The invention is suited to test multi target sequence.

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权利要求书 3 页 说明书 13 页 附图 4 页

[54] 发明名称

用于多重实时核酸扩增检测的探针编码方法

[57] 摘要

用于多重实时核酸扩增检测的探针编码方法，涉及一种核酸扩增检测，尤其是涉及一种用于多重实时核酸扩增的探针编码方法，提供一种使目前的多重实时核酸扩增可检测的靶序列数目明显多于目前仪器可检测的荧光染料数目的探针编码方法或探针组合方法。先将核酸扩增检测仪器检测的荧光染料作为荧光探针标记的基本元素，称荧光基本元素；荧光基本元素之间的相互组合标记靶序列的特异指示探针，互相组合是指组合编码、排列编码和指数编码。利用好荧光基本元素数目、循环阈值和各荧光基本元素相对强度这三个变量来产生出任意多个基因特异性荧光指纹图谱，以便在荧光基本元素数目有限的情况下仍能同时检测出许许多多不同核酸序列。适合于多种靶序列的检测。

1、一种用于多重实时核酸扩增检测的探针编码方法，其特征在于其步骤为：

1) 将核酸扩增检测仪器所能检测的不同荧光染料作为荧光探针标记的基本元素，称之为荧光基本元素；

2) 以荧光基本元素之间的相互组合来复合或混合标记靶序列的特异指示探针。

2、如权利要求 1 所述的用于多重实时核酸扩增检测的探针编码方法，其特征在于以荧光基本元素之间的相互组合而不只是单一的荧光基本元素来复合或混合标记靶序列的特异指示探针；将所标记的探针加入到实时核酸扩增体系并对待测核酸序列进行扩增；协同利用实时核酸扩增曲线图谱中出现的荧光基本元素的种类、循环阈值差别和各荧光基本元素相对强度差异这三个变量来区分、识别基因特异性荧光指纹图谱及其对应的靶序列，以便在荧光基本元素数目有限的情况下同时检测出多种不同核酸序列。

3、如权利要求 1 所述的用于多重实时核酸扩增检测的探针编码方法，其特征在于所说的荧光基本元素包括但并不局限于 ALEXA 350, FAM, HEX, TET, JOE, VIC, ROX, Texas Red, Cy5, Cy5.5, TAMRA 等。

4、如权利要求 1 所述的用于多重实时核酸扩增检测的探针编码方法，其特征在于所说的互相组合为组合编码，组合编码只考虑荧光基本元素种类的不同而忽略不同荧光基本元素间荧光强度的差异，可将荧光基本元素按数学的组合规则组合成不同的荧光标记基团或探针。

5、如权利要求 1 所述的用于多重实时核酸扩增检测的探针编码方法，其特征在于所说的互相组合为排列编码，排列编码即考虑荧光基本元素种类的不同又区分各荧光基本元素间的相对强弱，可按数学的排列规则组合成不同的荧光标记基团或探针。

6、如权利要求 1 所述的用于多重实时核酸扩增检测的探针编码方法，其特征在于所说的互相组合为指数编码，指数编码同时考虑荧光基本元素种类的差别、各元素间荧光强度的相对强弱及荧光强度大小差异的不同程度，可按数学上指数规则组合成不同的荧光标记基团或探针。

7、如权利要求 1 所述的用于多重实时核酸扩增检测的探针编码方法，其特征在于所说的荧光探针为可用于实时核酸扩增检测的荧光探针，它们选自 TaqMan™探针、TaqMan-MGB™探针、分子信标探针、荧光能量共振转移探针、置换探针、蝎子引物、Amplifier 引物；所

说荧光探针为依赖聚合酶 5' → 3' 外切核酸活性的探针如 TaqMan 探针, 或不依赖聚合酶 5' → 3' 外切核酸活性的探针。

8、如权利要求 7 所述的用于多重实时核酸扩增检测的探针编码方法, 其特征在于所说的不依赖聚合酶 5' → 3' 外切核酸活性的探针选自分子信标探针和置换探针。

9、如权利要求 1 所述的用于多重实时核酸扩增检测的探针编码方法, 其特征在于所说核酸扩增检测为多色实时核酸扩增检测, 或终点式的核酸扩增检测。

10、如权利要求 9 所述的用于多重实时核酸扩增检测的探针编码方法, 其特征在于所说的实时核酸扩增检测为实时核酸扩增检测的基因分型, 实时核酸扩增选自实时 PCR、实时 RT-PCR、Q-Beta 扩增、实时核酸序列依赖的扩增中的一种。

11、如权利要求 1 所述的用于多重实时核酸扩增检测的探针编码方法, 其特征在于所说的荧光基本元素的种类是指在实时核酸扩增曲线图谱中有那些荧光基本元素出现; 所说的循环阈值差别是指所出现的各荧光基本元素之间出峰时间的差别; 所说的荧光基本元素之间荧光强度大小差异程度是指每一荧光基本元素的大小水平, 或荧光水平, 或相对程度, 或相对强度组合成不同的元素。

12、如权利要求 1 所述的用于多重实时核酸扩增检测的探针编码方法, 其特征在于利用各种荧光基本元素出峰的相对时间、曲线形状区分多种靶序列共存时那种序列存在并加以扩增检测。

13、如权利要求 1 所述的用于多重实时核酸扩增检测的探针编码方法应用于多种靶序列的检测。

14、如权利要求 13 所述的用于多重实时核酸扩增检测的探针编码方法应用于检测样品中只可能含有多种靶序列中的至少一种的基因分型。

15、如权利要求 14 所述的用于多重实时核酸扩增检测的探针编码方法应用于检测对于同一基因或基因家族中不同等位基因的基因分型。

16、如权利要求 15 所述的用于多重实时核酸扩增检测的探针编码方法应用于检测对针对特定靶基因进行基因分型, 使用至少一对引物进行扩增, 而用至少 2 个探针检测引物中间序列达到基因分型的目的。

17、如权利要求 13 所述的用于多重实时核酸扩增检测的探针编码方法, 其特征在于在所说的多种靶序列的检测中, 当单色与多色探针共同编码多个等位基因时, 纯合子与杂合子的区分是通过调整多色探针中各荧光基本元素所标记的探针比例来使多色探针在检测纯合子时, 各色荧光曲线峰值基本持平; 当单色与多色探针所检测的两个等位基因共存于一

个杂合子时，单色探针中那一颜色荧光的峰值就会高于其它颜色荧光峰值。

18、如权利要求 16 所述的用于多重实时核酸扩增检测的探针编码方法，其特征在于所说的探针选自高特异性的、可区分只有单个碱基差别的靶序列特异性荧光探针。

用于多重实时核酸扩增检测的探针编码方法

技术领域

本发明涉及一种核酸扩增检测，尤其是涉及一种用于多重实时核酸扩增的探针编码方法，该方法使多重实时核酸扩增检测的靶序列数目大大多于仪器可检测的荧光染料数目。

背景技术

实时聚合酶链式反应(Real-time PCR)在基因定量、基因分型和单核苷酸多态性(SNPs)检测等方面正得到越来越广泛的应用，实时 PCR 最突出的优点是扩增检测同步进行，其它依赖核酸扩增的检测技术，无论检测的通量和操作如何，其简便程度都不及实时 PCR，这也是目前实时 PCR 在临床上得以普遍应用的根本原因。然而，实时 PCR 存在检测容量小的问题，即在一次反应中可同时检测的靶序列数目受仪器荧光检测通道数目的限制。目前最新的实时 PCR 仪器能同时检测 5 种不同荧光染料，按照传统的检测方式也至多能检测 5 种靶序列。这一问题严重制约了实时 PCR 在多位点依赖的基因分型中的应用。

实时 PCR 的简便性及其它诸多优点吸引人们不断努力去提高其检测容量。自 Kramer 小组(Vet, J.A.M., et al, PNAS 96, 6394-6399, 1999) 率先在四色荧光 PCR 仪器上实现四种靶序列的同时检测以来，多组分实时 PCR 检测技术已广泛应用。2000 年 Tyagi 等(Tyagi, S., et al, Nature Biotechnology 18, 1191-1196, 2000) 发明波长迁移型分子信标，克服了单一光源无法同时检测多个荧光染料的困难，El Hajj 等据此实现了 5 色探针检测结核杆菌的利富平耐药突变(El Hajj, H.H., Journal of Clinical Microbiology 39, 4131-4137, 2001)。

撇开实时 PCR 仪器，Lee 等(Lee, L.G., et al, Biotechniques 27, 342-349, 1999) 利用荧光光度计同步扫描 7 种荧光染料，实现了 6 种靶序列的同时检测。另有利用 PCR 扩增产物的熔点差异，在实时 PCR 扩增结束后，进行熔点曲线分析，以实现多个靶序列的同时检测和基因分型(Herrmann, M.G., et al, Clinical Chemistry 50, 982, 2004; Elenitoba-Johnson, K.S.J., et al, Nature Medicine 7, 249-253, 2001; Ririe, K.M., et al, Analytical Biochemistry 245, 154-160, 1997)。但该方法的局限性在于 PCR 产物的熔点范围狭窄，很难容下 5 个以上靶序列的同时存在与区分，而且容易受到引物二聚体

等非特异扩增的干扰。

发明内容

本发明的目的是针对多重实时核酸扩增检测容量小，即按传统的一种荧光基团标记一种探针的方法，在一次反应中可同时检测的靶序列数目受仪器荧光检测通道数目的限制的问题，提供一种使多重实时核酸扩增可检测的靶序列数目明显多于目前仪器可检测的荧光染料数目的探针编码方法或探针组合方法。

本发明的具体步骤为：

1、将核酸扩增检测仪器所能检测的不同荧光染料作为荧光探针标记的基本元素，称之为荧光基本元素；

2、以荧光基本元素之间的相互组合来复合或混合标记靶序列的特异指示探针，所说的互相组合是指组合编码、排列编码和指数编码。

所说的组合编码只考虑荧光基本元素种类的不同而忽略不同荧光基本元素间荧光强度的差异，可以将荧光基本元素按数学的组合规则组合成不同的荧光标记基团或探针。所说的排列编码即考虑荧光基本元素种类的不同又区分各荧光基本元素间的相对强弱，可按数学的排列规则组合成不同的荧光标记基团或探针。所说的指数编码同时考虑荧光基本元素种类的差别、各元素间荧光强度的相对强弱、及荧光强度大小差异的不同程度，可按数学上指数规则组合成不同的荧光标记基团或探针。

所说的以荧光基本元素之间的相互组合而不只是单一的荧光基本元素来复合或混合标记靶序列的特异指示探针；将所标记的探针加入到实时核酸扩增体系并对待测核酸序列进行扩增；协同利用实时核酸扩增曲线图谱中出现的荧光基本元素的种类、循环阈值（ C_t 值）差别和各荧光基本元素相对强度差异这三个变量来区分、识别基因（信号）特异性荧光指纹图谱及其对应的靶序列，以便在荧光基本元素数目有限的情况下同时检测出多种不同核酸序列。所说的荧光基本元素包括但并不局限于 ALEXA 350, FAM, HEX, TET, JOE, VIC, ROX, Texas Red, Cy5, Cy5.5, TAMRA 等。

所说的荧光探针是指可用于实时核酸扩增检测的荧光探针，它们包括 TaqMan™探针、TaqMan-MGB™探针、分子信标探针（Molecular Beacons）、荧光能量共振转移探针（又称 LightCycler™探针）、置换探针、蝎子引物（Scorpions）、Amplifier 引物等。这些荧光探针可以分为依赖聚合酶 5' → 3' 外切核酸活性的探针如 TaqMan 探针，以及不依赖聚合酶 5' → 3' 外切核酸活性的探针，如包括分子信标探针和置换探针等。

所说核酸扩增检测为多色实时核酸扩增检测，或终点式的核酸扩增检测。

所说的荧光基本元素的种类是指在实时核酸扩增曲线图谱中有那些荧光基本元素出现；所说的循环阈值（ C_t 值）差别是指所出现的各荧光基本元素之间出峰时间的差别；所说的荧光基本元素之间荧光强度大小差异程度是指每一荧光基本元素的大小水平，或荧光水平，或相对程度，或相对强度组合成不同的元素。可利用各种荧光基本元素出峰的相对时间、曲线形状、有无曲线增长拐点等来区分多种靶序列共存时那种序列存在并加以扩增检测。

本发明所提供的编码方法适合于多种靶序列的检测，尤其适合于检测样品中只可能含有多种靶序列中的至少一种（经常是一种或少数几种）的基因分型，特别是对于同一基因或基因家族中不同等位基因的基因分型。本发明所提供的编码方法特别适合针对特定靶基因进行基因分型，此时可以使用至少一对（经常是一对或少数几对）引物进行扩增，而用至少2个探针检测引物中间序列达到基因分型的目的。

所说的探针优选不依赖聚合酶 5' → 3' 外切核酸活性的探针，如包括分子信标探针和置换探针等。

所说的实时核酸扩增检测为实时核酸扩增检测的基因分型，实时核酸扩增选自实时PCR、实时 RT-PCR、Q-Beta 扩增、实时核酸序列依赖的扩增中的一种。

当单色与多色探针共同编码多个等位基因时，纯合子与杂合子的区分是通过调整多色探针中各荧光基本元素所标记的探针比例来使多色探针在检测纯合子时，各色荧光曲线峰值基本持平。这样在单色与多色探针所检测的两个等位基因共存于一个杂合子时，单色探针中那一颜色荧光的峰值就会高于其它颜色荧光峰值。

所说的探针优选高特异性的、可以区分只有单个碱基差别的靶序列特异性荧光探针。这种探针适合于各种靶序列差异的识别，而且大量探针共存并不会造成彼此之间的干扰，只有与探针完全匹配的靶序列结合才可以引起探针荧光强度的增加。

总之，本发明的主旨就是要协同利用好荧光基本元素数目、循环阈值（ C_t 值）和各荧光基本元素相对强度这三个变量来产生出任意多个基因（信号）特异性荧光指纹图谱，以便在荧光基本元素数目有限的情况下仍能同时检测出许许多多不同核酸序列。

由此可见，按照探针编码方法，无论采用组合编码、排列编码还是指数编码，都可得到远多于荧光基本元素的编码数，从而可以检测种类更多的靶序列。

可把用一种荧光染料（亦即一个荧光基本元素）标记的探针称作单色荧光探针或者单色探针。类似地，可把两个或两个以上荧光染料（亦即多个荧光基本元素）复合或混合标记的探针，称作复合多色荧光探针或多色探针（参见图 1）。无论单色探针还是多色探针都是针对各自的靶序列设计，也就是说它们都可以检测各自的靶序列。单色探针或者多色探针的

序列可以相同也可以不同,但多数情况下是相同的。

本发明适合于基于实时核酸扩增检测的基因分型,实时核酸扩增包括实时 PCR、实时 RT-PCR、实时核酸序列依赖的扩增(NASBA)等。本发明当然并不排除用于终点式的核酸扩增检测,但是,发明人认为,实时检测更能充分发挥本发明的优势。首先,实时检测方式属于动力学检测,即使大量探针共存造成背景信号增加,也不会对动力学检测特性造成明显影响。其次,实时核酸扩增检测如实时 PCR 检测的反应曲线的循环阈值(G)由模板浓度决定,并具有极好的重现性,对于采用同一序列设计的多色探针而言,各个荧光染料所展示的扩增曲线应具有相同或者至少相近的 G 值。另外,对于排列编码和指数编码的多色探针而言,判断结果所依据的是同一反应中多色探针显示的多个实时扩增曲线之间峰值的相对位置与强弱(荧光指纹图谱)。采用同一序列的多色探针,虽然荧光染料不同,所展示的与模板杂交的效率应相同,而且受外界影响的程度也相同,因此,同一序列的多色探针实时扩增曲线的最终荧光强度之比也将是相对固定的。这就使我们能够利用每一组合多色探针与其特异性序列杂交所产生的特异性荧光指纹图谱来区分 PCR 产物中有那种特异性序列存在并得以扩增。

本发明所述探针编码方法虽然侧重应用于核酸检测,但任何一个懂得基本常识与技术的业内人士都可按本发明的想法把它推广到其它荧光检测体系乃至非荧光编码体系,实现利用有限几个元素来编码无限多个特征标记物来。因此,该发明涵盖但不应局限在核酸检测领域。

由此可见,本发明与目前通用的用一种荧光基本元素来标记一种靶序列特异探针的方式相比,相互组合的方式可以标记更多的特异指示探针,从而可以检测更多的靶序列。

附图说明

图 1 为多色探针原理图。在图 1 中, FAM(空心圆)和 ROX(实心圆)分别是两种荧光染料,编码分别是 1000 和 0100,它们相互混合后,可以认为形成了一种新的染料类型,即混合染料,如图 1 中的 FAM/ROX(空心 and 实心各半的圆),编码是 1100。类似地,如果同一种探针分别使用了两种染料作为标记,如图 1 中的探针分别标记了 FAM(空心圆)和 ROX(实心圆),二者混合后,就相当于形成了一种标记混合染料的探针,此处是双色探针。该探针标记的是 FAM/ROX(空心 and 实心各半的圆,编码是 1100)。灰色圆表示置换探针的淬灭剂。

图 2 为 8 株(菌株 1~8)蜡样芽孢杆菌标准菌株的实时 PCR 分型。在图 2 中,荧光检测同时在 Cy5(实心圆球)、ROX(空心圆球)、HEX(实心三角)和 FAM(空心三角)四个通道中进行。其横坐标为循环数(Cycle Number),纵坐标为荧光值(Fluorescence)。

图 3 为 HPV 15 个基因型的分型结果。在图 3 中, 荧光检测同时在 Cy5 (实心圆球)、ROX (空心圆球)、HEX (实心三角) 和 FAM (空心三角) 四个通道中进行。其横坐标为循环数 (Cycle Number), 纵坐标为荧光值 (Fluorescence)。

图 4 为 HCV 6 个基因型的实时 RT-PCR 分型体系。在图 4 中, 荧光检测同时在 Cy5 (实心圆球)、ROX (空心圆球)、HEX (实心三角) 和 FAM (空心三角) 四个通道中进行。其横坐标为循环数 (Cycle Number), 纵坐标为荧光值 (Fluorescence)。

具体实施方式

以下实施例将结合附图对本发明作进一步的说明, 实际上以下的实施例只是说明本发明, 但显然并不限于这些应用, 业内人士完全可以按照本发明的原理推广应用于多个领域。

实施例 1: 探针编码方法用于蜡样芽孢杆菌的基因分型。

蜡样芽孢杆菌是一种食源性致病菌, 可引起感染性腹泻, 也是引发细菌性食物中毒最常见的一种条件致病菌。如何及时确定传染源, 是控制和降低此类细菌食物中毒发生的关键。目前, 蜡样芽孢杆菌食物中毒的溯源仍以传统的生化分型为主, 但生化分型耗时较长, 结果不稳定, 缺乏重现性, 且不能对所有菌株进行分型, 因此无法及时溯源。

本实施例中用以分型的 *vrrA* 基因是炭疽芽孢杆菌的一个多态性位点, 具有串联重复序列和高变区, 由于蜡样芽孢杆菌和炭疽芽孢杆菌同属芽孢杆菌, 故以 *vrrA* 基因作为分子遗传标记来研究蜡样芽孢杆菌的多态性, 采用实时 PCR 技术和组合探针编码技术进行基因分型。

首先根据 8 个标准菌株的 *vrrA* 基因序列设计荧光双链置换探针, 每个菌株均有一套特异性的探针, 分别标记 FAM, HEX, ROX, CY5 四种荧光染料的不同组合, 并根据二进制为其编码 (见表 1)。然后用一对文献报道的引物进行 PCR 扩增, 产物为 230bp, 引物序列为: Sense: 5'-ACTACCACCAATGGCACA-3', Anti-sense: 5'-GCTGCATGTATGGTTGAT-3'。

在同一 PCR 反应管中, 加入所有探针, 反应总体积为 25 μ L, 内含 10 $\times$ 缓冲溶液 2.5 μ L, 3.0 mM MgCl₂, 0.25 mM dNTP, 1.0 U Taq 酶, 20 pmol 上游引物, 2.5 pmol 下游引物, 探针各 20 pmol, 5 μ L 菌液模板。反应条件: 95 $^{\circ}$ C 3min, 第一个循环周期为 94 $^{\circ}$ C 15s, 52 $^{\circ}$ C 20s, 72 $^{\circ}$ C 20s, 共 10 个循环, 第二个循环周期为 94 $^{\circ}$ C 15s, 52 $^{\circ}$ C 20s, 72 $^{\circ}$ C 20s, 28 $^{\circ}$ C 18s, 共 40 个循环, 荧光在第二个循环周期的 28 $^{\circ}$ C 时采集。利用 MX 3000P 实时 PCR 仪进行检测。实验中为了提高探针的荧光效率和杂交效率, 采用低熔点探针和不对称 PCR 方式进行; 最后根据实时荧光 PCR 结果 (荧光曲线图谱差别), 可以将 8 种标准菌株成功地区分成 8 种亚型 (见图 2)。

实施例 2：人乳头瘤病毒（HPV）的基因分型。

HPV 是一组高度特异的 DNA 病毒，病毒核酸为双链环状 DNA，含有 8000 碱基对，属乳多空病毒科 A 亚组，直径约 55 nm。HPV 对生殖道鳞状上皮有强嗜性，各种 HPV 基因结构大致相似，可分为 3 个部分：晚期区、早期区和调节区。HPV 与多种疾病的发生有关，严重的可以致癌，如宫颈癌、皮肤癌等。根据 HPV 与肿瘤发生的关系，可将其分为高危型和低危型两类。本实施例以 15 种不同型别的 HPV 作为实验对象，根据其 L1 区的基因序列设计荧光双链置换探针，每种型都设计一套特异性的探针（表 2），标记 FAM，HEX，ROX，CY5 四种荧光染料的全部组合，并以二进制编码。用一对通用引物 GP5+/GP6+ 进行 PCR 扩增，产物约 150bp，引物序列为 GP5+：5'-TTTGTACTGTGGTAGATACTAC-3'，GP6+：5'-GAAAAATAAACTGTAAATCATA-TTC-3'。在一个 PCR 管中，加入所有探针，反应总体积为 25 μ L，内含 50 mM KCl，2.5 mM MgCl₂，0.25 mM dNTP，1.0U Taq 酶，上下游引物各 0.2 μ M，每种探针 0.2 μ M，5 μ L 质粒模板。反应条件为：95 $^{\circ}$ C 3min，循环周期为 94 $^{\circ}$ C 15s，45 $^{\circ}$ C 20s，72 $^{\circ}$ C 20s，共 40 个循环，在退火时采集荧光。用 Rotor-Gene 3000 实时 PCR 仪进行检测。最后根据实时荧光 PCR 结果（荧光曲线图谱的差别），成功地区分开 15 种不同亚型的 HPV 病毒（见图 3）。

实施例 3：HCV 具有高度的变异性，各型广泛分布于全世界范围内，同时特定地区的流行基因型也在其它地区发现，使 HCV 基因型分布复杂，加之 HCV 基因型分型手段的不断完善，使基因型分布的报道也频繁出现。本实施例以中国的 6 种主要亚型：1b，2a，3a，1a，3b 和 6a 为对象，按照探针编码原理进行分型。针对 HCV 的 6 个基因型，只需选择 3 种荧光染料，构成 FAM、HEX、ROX、FAM+HEX、HEX+ROX、FAM+ROX 六种组合。探针序列如表 3。中国地区 HCV 常见 6 个基因型进行编码而区分开。

利用通用引物进行 RT-PCR 扩增，上游引物 5' -CCCICGITTGGGTGTGCG-3'，下游引物：5' -GTAGGGTATCGATGACITTAC-3'，其中 I 为次黄嘌呤核苷酸，该通用引物 PCR 扩增产物长度为 256_bp。用通用引物在同一反应管中进行 RT-PCR 基因分型，反应总体积为 30 μ L，内含 50 mM KCl，2.5 mM MgCl₂，0.25 mM dNTP，1.0 UTaq 酶，12 U M-MLV 逆转录酶，20 U RNAsin，0.2 μ M 上游引物，0.2 μ M 下游引物，各个探针 0.2 μ M，加入 5 μ L 经 95 $^{\circ}$ C，5min 热裂解各基因型假病毒作为 RNA 模板。反应条件：42 $^{\circ}$ C，40min，95 $^{\circ}$ C 3min，循环周期为 95 $^{\circ}$ C 15s，50 $^{\circ}$ C 20s，72 $^{\circ}$ C 20s，共 40 个循环，荧光信号在 50 $^{\circ}$ C 时采集。最后根据实时荧光 PCR 结果，可以成功地区分开 6 种不同的 HCV 亚型（见图 4）。

实施例 4：用于多重实时核酸扩增检测的探针组合编码方法。设有 A 元素和 B 元素，可以分别构成 A、B 和 A+B 三种组合；同样如设有 A、B 和 C 三元素，可以构成 A、B、C、A

+B、A+C、B+C、A+B+C 共七种组合。因此，按照数学上的组合规则，对于 N 个荧光基本元素，共有 $C_N^1 + C_N^2 + \dots + C_N^N = \sum C_N^i (i=1 \sim N) = 2^N - 1$ 种组合方式，其中的任一个都可以标记一种靶序列特异探针，故可得到 $2^N - 1$ 种不同标记的荧光探针。这样就可以检测 $2^N - 1$ 种不同的靶序列。按这一方案，四色荧光 PCR 仪器（共可同时检测四种荧光基本元素）就可检测 $2^4 - 1 = 15$ 种靶序列，五色荧光 PCR 仪器（共可同时检测五种荧光基本元素）就可检测 $2^5 - 1 = 31$ 种基因型。

根据组合编码方法可以看出，目前的测定方式实际上仅仅用单一的荧光基本元素逐个进行探针标记，并没有考虑它们之间的组合，所编码的探针只能等于荧光基本元素的个数。因此，对于四色荧光 PCR 仪器（共可检测四种荧光基本元素），就只能编码四个荧光探针，检测四种靶序列。同样，五色荧光 PCR 仪器（共可检测五种荧光基本元素）就只能同时检测五种靶序列。

实施例 5：用于多重实时核酸扩增检测的探针排列编码方法。组合编码方法只考虑了荧光基本元素种类的不同组合情况而没有考虑各荧光基本元素之间相对强度是否相同，如果进一步考虑组合内部荧光基本元素的量上的差异，就可以用不同的荧光强弱组成不同的排列，比如，同样是由 A+B 构成的组合编码，可以根据他们之间荧光强度的高低不同区别为 A>B 或 A<B 两种情况（虽然 A=B 代表第三种情况，但由于要使 PCR 反应中两种或两种以上荧光基本元素的强度相同非常难以控制，并且 A=B 时 AB 与 BA 不能区分，不能以排列方式来表述，故将此种情况不在排列编码范围内预与考虑），用这两种情况分别表示两种探针，就可以得到两种编码。同样由 A+B+C 组合的组合编码，可以根据荧光强度大小差异组成 A>B>C、A>C>B、B>A>C、B>C>A、C>A>B、C>B>A 等 6 种情况，可以得到 6 种编码。这种组合方式符合数学的排列规则，因此，对于 N 个荧光基本元素，就可以获得 $\sum P_N^i (i=1 \sim N)$ 个不同的荧光基本元素的组合。其中的任一个都可以标记一种靶序列特异探针，这样就可以检测 $\sum P_N^i (i=1 \sim N)$ 种不同的靶序列。按照排列编码，四色荧光 PCR 仪器可以同时检测 $P_4^1 + P_4^2 + P_4^3 + P_4^4 = 64$ 种靶序列，五色荧光 PCR 仪器可以同时检测 $P_5^1 + P_5^2 + P_5^3 + P_5^4 + P_5^5 = 325$ 种靶序列。

实施例 6：用于多重实时核酸扩增检测的探针指数编码方法。不难看出，排列编码方法只考虑了荧光基本元素之间存在大小差异，并没有考虑大小差异的程度。如果我们把每一种荧光基本元素的大小分成若干个水平，称为荧光水平，并用 x 表示荧光水平数，假定不同荧光基团的 x 值相等，则对于 N 色荧光 PCR 仪器，可以设计出的探针数目为： $P_N^1 + \sum x^i P_N^i (i=2 \sim N)$ 个荧光探针（该公式将单色荧光探针水平数均计为 1，这是考虑了荧光强度本身就是相对

的这一性质)。按照这一规则,如果一个染料荧光水平数 $x=10$,那么,四色荧光 PCR 仪器可以同时检测 $P_4^1 + (10^2 P_4^2 + 10^3 P_4^3 + 10^4 P_4^4) = 265,204$ 种基因型,由于理论上每一种荧光染料的荧光水平数可以任意多个,因此指数编码理论上可以设计出任意多个编码方案。

实施例 7: 本发明所说的荧光探针,是指可用于实时核酸扩增检测的荧光探针,它们包括 TaqMan™探针、TaqMan-MGB™探针、分子信标探针 (Molecular Beacons)、荧光能量共振转移探针 (又称 LightCycler™探针)、置换探针、蝎子引物 (Scorpions)、Amplifier 引物等。这些荧光探针可以分为依赖聚合酶 5'→3' 外切核酸活性的探针如 TaqMan 探针,以及不依赖聚合酶 5'→3' 外切核酸活性的探针,如包括分子信标探针和置换探针等。

实施例 8: 本发明优选采用不依赖聚合酶 5'→3' 外切核酸活性的探针,如包括分子信标探针和置换探针等。因为这些探针可用于所有实时核酸扩增检测的模式,而且反应条件不受依赖核酸聚合酶 5'→3' 外切酶活性的限制。

实施例 9: 本发明优选采用高特异性的荧光探针,这类探针可以区分只有单个碱基差别的靶序列。适合各种靶序列差异的识别,而且大量探针共存并不会造成彼此之间的干扰。因为可以区分单个核苷酸差别的荧光探针,只有与探针完全匹配的靶序列结合才可以引起探针荧光强度的增加。

实施例 10: 本发明提供的编码方法适合多种靶序列的检测,尤其适合检测样品中只可能含有多种靶序列中的一种(或少数几种)的基因分型。特别是对于同一基因(或基因家族)中不同等位基因的基因分型,由于可以使用一对或少数几对引物进行扩增,用编码的荧光探针识别不同的引物间序列,可以在同一管内混合所有的探针。采用一对或少数几对引物产生非特异扩增的机会较少,反应条件更容易优化,这对于大量探针存在的反应体系来说,意味着反应条件的优化可以集中于探针上,使检测体系的建立过程更为简单。

实施例 11: 本发明特别适合针对特定靶基因进行基因分型,此时可以使用一对或少数几对引物进行扩增,而用多个探针检测引物中间序列达到基因分型的目的。比如对于人乳头瘤病毒 (HPV) 的实时 PCR 分型,对于免疫缺陷病毒 (HIV) 的实时 RT-PCR 分型,对于丙型肝炎病毒的实时 RT-PCR 分型,对于细菌的 16S 核糖体 RNA 的实时 PCR 分型,或者针对某一种细菌的实时 PCR 分型,包括耐药突变分型等。

实施例 12: 本发明用于对特定靶基因进行基因分型时,对于可能出现的同一标本中有两种或者两种以上亚型混合共存的情况,可根据两种探针的实时 PCR 曲线的 C_t 值、相对荧光强度以及扩增曲线的形状进行区分。对于 FAM 和 HEX 编码的双色探针(编码同一靶序列)而言,当反应中存在该双色探针的对应的靶序列时,仪器上 FAM 和 HEX 通道上显示的两条

实时 PCR 曲线的 G 差值 (ΔG) 理论上应为零, 并且二者的荧光强度比例一定。若同时出现 FAM 单色探针和 HEX 单色探针编码的两种不同靶序列, 则出现二者的实时扩增曲线之 ΔG 或者为正或者为负的情况, 而且二者荧光强度之比也必不同于双色探针的情况。只有当双色探针检测的靶序列与两种单色探针所检测的靶序列的起始靶序列含量恰好相等 (如杂合子中的两种不同等位基因, 此时出现 $\Delta G=0$), 并且仪器检测的二者的荧光强度比恰好等于双色探针的荧光比时, 才会造成二者不可分辨的结果, 显然, 后两种情况同时发生的概率极低。因为混合样品中亚型含量完全相等的可能性已经很小, 荧光探针的荧光强度受多种因素影响, 要使两个单色探针荧光强度之比恰好等于双色探针荧光比的可能性更小。正常的实时 PCR 扩增曲线属于典型的 S 型, 不会发生突然增加的情况 (即出现拐点)。然而, 如果发生单色探针和双色探针分别识别的靶序列共存的情况, 比如 FAM 和 FAM/HEX 分别识别的靶序列共存, FAM 的扩增曲线实际上反映的是两扩增曲线之和, 比如曲线的前半部分是单色探针 FAM 的情况, 而后半部分则既有 FAM 单色探针荧光水平增长的情况又有 FAM/HEX 双色探针中 FAM 的增长情况, 从而在曲线中出现增长拐点; 无论这两种靶序列的浓度孰大, 都会在 FAM 扩增曲线中出现拐点。只不过在单色探针检测的靶序列浓度较高时, FAM 扩增曲线先出现而 HEX 扩增曲线随后出现并且 FAM 扩增曲线拐点必然出现在 HEX 曲线开始升高的附近, 因为拐点的位置也是 HEX 增加的位置。反之, 如果双色探针所检测的靶序列浓度较高则 FAM 与 HEX 扩增曲线首先同时出现, 但 FAM 曲线随后会出现一个增长拐点而 HEX 曲线为典型的 S 型曲线, 不出现拐点。

因此, 对于出现两色扩增曲线的体系, 就很容易根据曲线之间的关系和形状 (荧光指纹图谱) 进行判断。以反应中同时出现 FAM 和 HEX 扩增曲线为例, 体系中的靶序列可能有 5 种情况: 只存在双色探针 FAM/HEX 识别的靶序列, 同时存在 FAM 单色探针和 HEX 单色探针识别的两种靶序列, 同时存在 FAM 单色探针和双色探针 FAM/HEX 识别的两种靶序列, 同时存在 HEX 单色探针和双色探针 FAM/HEX 识别的两种靶序列, 同时存在 FAM 单色探针、HEX 单色探针、双色探针 FAM/HEX 识别的三种靶序列。如果两条曲线属典型的 S 型扩增曲线, 且 $\Delta G=0$, 且两者的荧光强度比与双色探针的扩增曲线一致, 就可以直接判断为只存在双色探针 FAM/HEX 识别的靶序列。如果是彼此分离的两条扩增曲线, 且是典型的 S 型扩增曲线, 就可以直接判断为同时存在 FAM 和 HEX 单色探针分别识别的两种靶序列。若两条扩增曲线中, FAM 的扩增曲线出现新的增长拐点, 则是同时存在 FAM 单色探针和双色探针 FAM/HEX 识别的两种靶序列。如果两条扩增曲线中, HEX 的扩增曲线出现新的增长拐点, 则可以判断为是同时存在 HEX 单色探针和双色探针 FAM/HEX 识别的两种靶序列。如果两条扩增曲线中 FAM

和 HEX 的扩增曲线均出现新的增长拐点,则可以判断为同时存在 FAM 单色探针、HEX 单色探针、及双色探针 FAM/HEX 识别的三种靶序列。

当然,对于大容量基因分型情况,干扰的概率会增加。但只要我们充分利用好荧光基本元素数目、 C_t 值、及各荧光基本元素的相对强度这三个变量,便可生成许许多多基因型特异性荧光指纹图谱。再通过充分掌握临床资料,适当改变探针标记策略等手段,就完全有可能排除共存亚型引起的干扰。

实施例 13: 当本发明用于检测一个样品中可能同时存在多个等位基因中的一种(纯合子)或两种(杂合子)的情况时,如果一个等位基因用了单色(如红色)探针来检测而另一个等位基因用了包含上述单色探针颜色的双色或多色探针来检测,则此时由于 $\Delta C_t=0$,对杂合子与纯合子的区分必需依靠荧光强度的变化。为了便于区分,此时有必要调整双色或多色探针中各色荧光基本元素所标记的探针比例,从而使双色或多色探针检测纯合子样品时各色荧光曲线的峰值基本相等;这样,当遇到单色(如红色)探针与双色(如红、绿)或多色(如红、绿、黄)探针所能检测的不同等位基因共存于一个杂合子时,红色探针虽与其它颜色的探针同时出峰,但其峰值必然会高于其它颜色的荧光峰值,从而把单色探针所检测的纯合子(只有一色荧光出现)及双色或多色探针所检测的纯合子(有两色或多色曲线同时出峰并且各曲线峰值基本持平)与单色——双色(多色)探针共同检测的杂合子(有两色或多色曲线同时出峰但某色曲线峰值会明显高于其它曲线峰值)区分开来。利用相同的原理,不同的多色探针所检测的纯合子与杂合子也同样能够区分开来。

核苷酸序列表

表 1: vrrA 基因分型中的探针序列及编码

菌株	探针	探针编码(代码)
菌株 1	5'-FAM-CCGCCCCAAT-PO ₄ -3' 5'-TTGGGGCGG-Dabcyl-3'	FAM (1000)
菌株 2	5'-HEX-TTTCGCCACAATATC- PO ₄ -3' 5'-ATATTGTGGCG-Dabcyl-3'	HEX (0100)
菌株 3	5'-ROX-CAGCACCAGCA-PO ₄ -3' 5'-GCTGGTGCTG-Dabcyl-3'	ROX (0010)
菌株 4	5'-Cy5-CGCCGCAATATC- PO ₄ -3' 5'-ATATTGCGGCG-BHQ-3'	Cy5 (0001)
菌株 5	5'-FAM-CCAACACCAATAC-PO ₄ -3' 5'-TATTGGTGTGG-Dabcyl-3' 5'-ROX-CAGCACCAGCA-PO ₄ -3' 5'-GCTGGTGCTG-Dabcyl-3'	FAM+ROX (1010)
菌株 6	5'-FAM-CCAACACCAATAC-PO ₄ -3' 5'-TATTGGTGTGG-Dabcyl-3' 5'-Cy5-ACCGCCAATGA-PO ₄ -3' 5'-CATTGGCGGT-BHQ-3'	FAM+Cy5 (1001)
菌株 7	5'-ROX-CGAGCCATTTATG- PO ₄ -3' 5'-ATAAATGGCTCG-Dabcyl-3' 5'-CY5-CGCCGCAATATC- PO ₄ -3' 5'-ATATTGCGGCG-BHQ1-3'	ROX+Cy5 (0011)
菌株 8	5'-FAM-CCAACACCAATAC-PO ₄ -3' 5'-TATTGGTGTGG-Dabcyl-3' 5'-ROX-CAGCACCAGCA-PO ₄ -3' 5'-GCTGGTGCTG-Dabcyl-3' 5'-Cy5-ACCGCCAATGA-PO ₄ -3' 5'-CATTGGCGGT-BHQ1-3'	FAM+ROX+Cy5 (1011)

表 2 HPV 亚型特异性探针及其编码

型别	探针序列	编码
HPV18	5'-FAM-TCTCCTGTACCTGGG-PO ₄ -3'	FAM
	5'-CAGGTACAGGAGA-DABCYL-3'	(1000)
HPV6	5'-HEX-TTCCTTCCACATACACCA-PO ₄ -3'	HEX
	5'-GTGTATGTGGAAGA-DABCYL-3'	(0100)
HPV16	5'-ROX-TGCTGCCATATCTACT-PO ₄ -3'	ROX
	5'-TAGATATGGCAGCA-DABCYL-3'	(0010)
HPV11	5'-CY5-CTGTGTCTAAATCTGCT-PO ₄ -3'	CY5
	5'-CAGATTTAGACACACAG-BHQ-3'	(0001)
HPV35	5'-FAM, HEX-AATCTGTGTGTCTGCT-PO ₄ -3'	FAM+HEX
	5'-CAGAACACACAGA-DABCYL-3'	(1100)
HPV31	5'-FAM, ROX-CAATTGCAAACAGTGAT-PO ₄ -3'	FAM+ROX
	5'-CACTGTTTGCAATTG-DABCYL-3'	(1010)
HPV33	5'-FAM, CY5-TGACTTTATGCACACA-PO ₄ -3'	FAM+CY5
	5'-TGTGCATAAAGTCA-BHQ-3'	(1001)
HPV52	5'-HEX, ROX-TTCTTTATGTGCTGAGGT-PO ₄ -3'	HEX+ROX
	5'-CTCAGCACATAAAG-DABCYL-3'	(0110)
HPV58	5'-HEX, CY5-AACACTGAAGTAACTAAGGA-PO ₄ -3'	HEX+CY5
	5'-CTTAGTTACTTCAGTG-BHQ-3'	(0101)
HPV42	5'-ROX, CY5-ACTGCAACATCTGGT-PO ₄ -3'	ROX+CY5
	5'-CAGATGTTGCAGT-BHQ-3'	(0011)
HPV43	5'-FAM, HEX, ROX-AAGACCCTACTGTGCC-PO ₄ -3'	FAM+HEX+OX
	5'-CACAGTAGGGTC-DABCYL-3'	(1110)
HPV44	5'-FAM, HEX, CY5-AACCACTACACAGTCCC-PO ₄ -3'	FAM+HEX+CY5
	5'-GACTGTGTAGTGG-BHQ-3'	(1101)
HPV39	5'-FAM, ROX, CY5-CCTCTATAGAGTCTTCCA-PO ₄ -3'	FAM+ROX+CY5
	5'-GAAGACTCTATAGAGG-BHQ-3'	(1011)
HPV45	5'-HEX, ROX, CY5-CCCTAATTTAATATTATGTGCC-PO ₄ -3'	HEX+ROX+CY5
	5'-CACATAATGTTAAATTAG-BHQ-3'	(0111)
HPV40	5'-FAM, HEX, ROX, CY5-AAACACAGTCCCCCA-PO ₄ -3'	FAM+HEX+ROX+
	5'-GGGGACTGTGT-BHQ-3'	CY5(1111)

表 3: HCV 亚型特异性探针及其编码

名称	探针序列	编码
1b-S	5'-ROX-GGAAGGCGACAACCTA-PO ₄ -3'	ROX (001)
1b-A	5'-GTTGTCGCCTTCC -DABCYL-3'	
2a-S	5'-FAM-GATCGGCGCTCCAC-PO ₄ -3'	FAM (100)
2a-A	5'-GGAGCGCCGATC-DABCYL-3'	
3a-S	5'-HEX-TACGTA [□] AAACTTCTGAACGGTC-PO ₄ -3'	HEX (010)
3a-A	5'-CGTTCAGAAGTTTACG-DABCYL-3'	
1a-S	5'-ROX/HEX-TCCGAGGTAGACGTCAGC-PO ₄ -3'	ROX + HEX (011)
1a-A	5'-TGACGTCTACCTCG-DABCYL-3'	
3b-S	5'-FAM/HEX-CTAGTACGTAAGACTTCCGAG-PO ₄ -3'	FAM+HEX (110)
3b-A	5'-GGAAGTCTTACGTACT-DABCYL-3'	
6a-S	5'- ROX/FAM-AGCCCAGAGGCAGG-PO ₄ -3'	ROX+FAM (101)
6a-A	5'- TGCCTCTGGGCT-DABCYL-3'	

注：字符带边框的为与模板不匹配碱基，这样可以在杂交时减少碱基对荧光素 HEX 的淬灭作用。

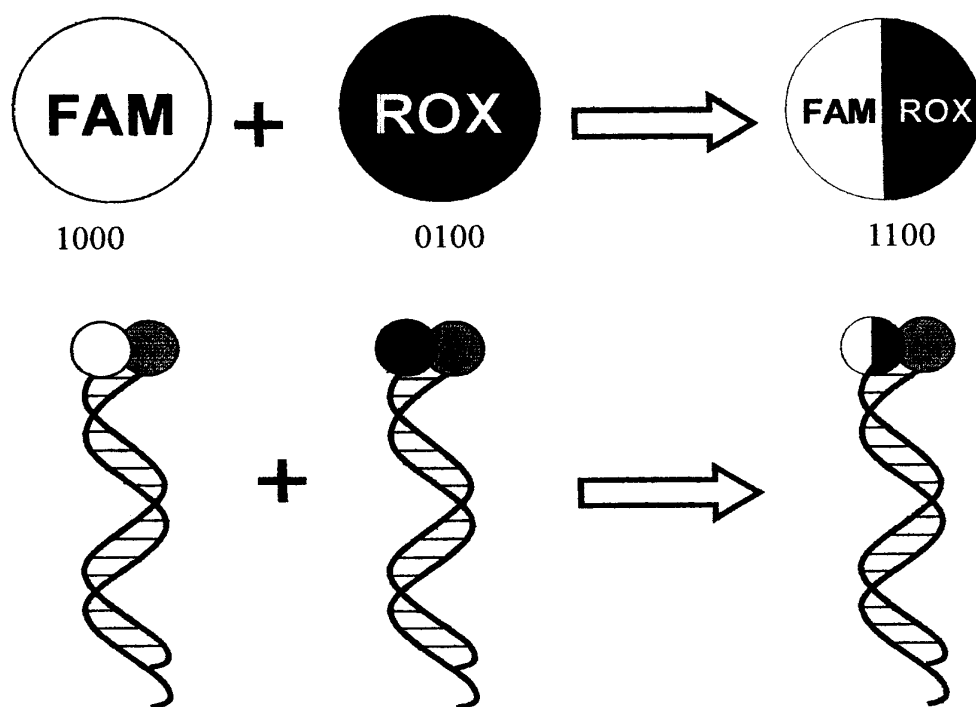


图 1

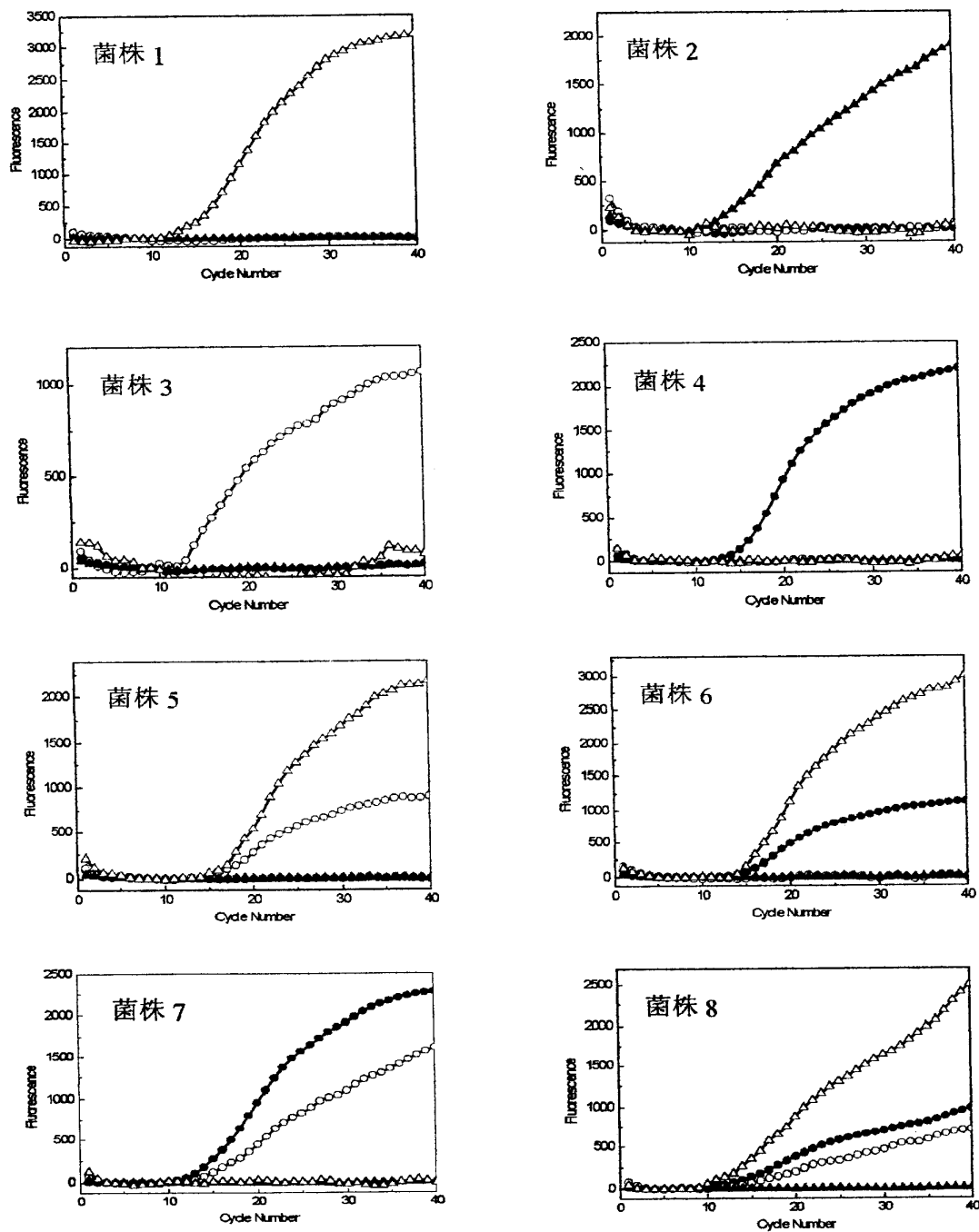


图 2

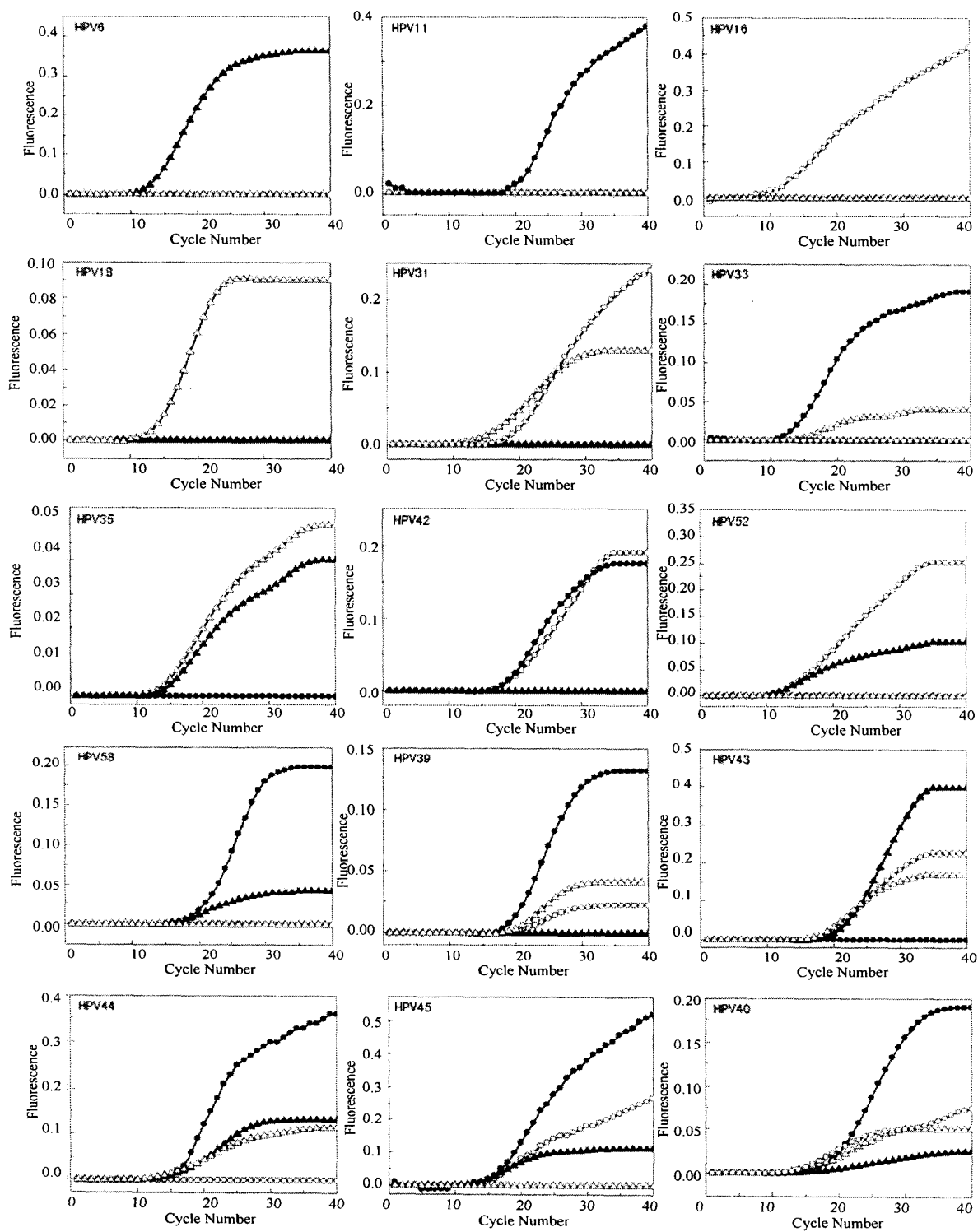


图 3

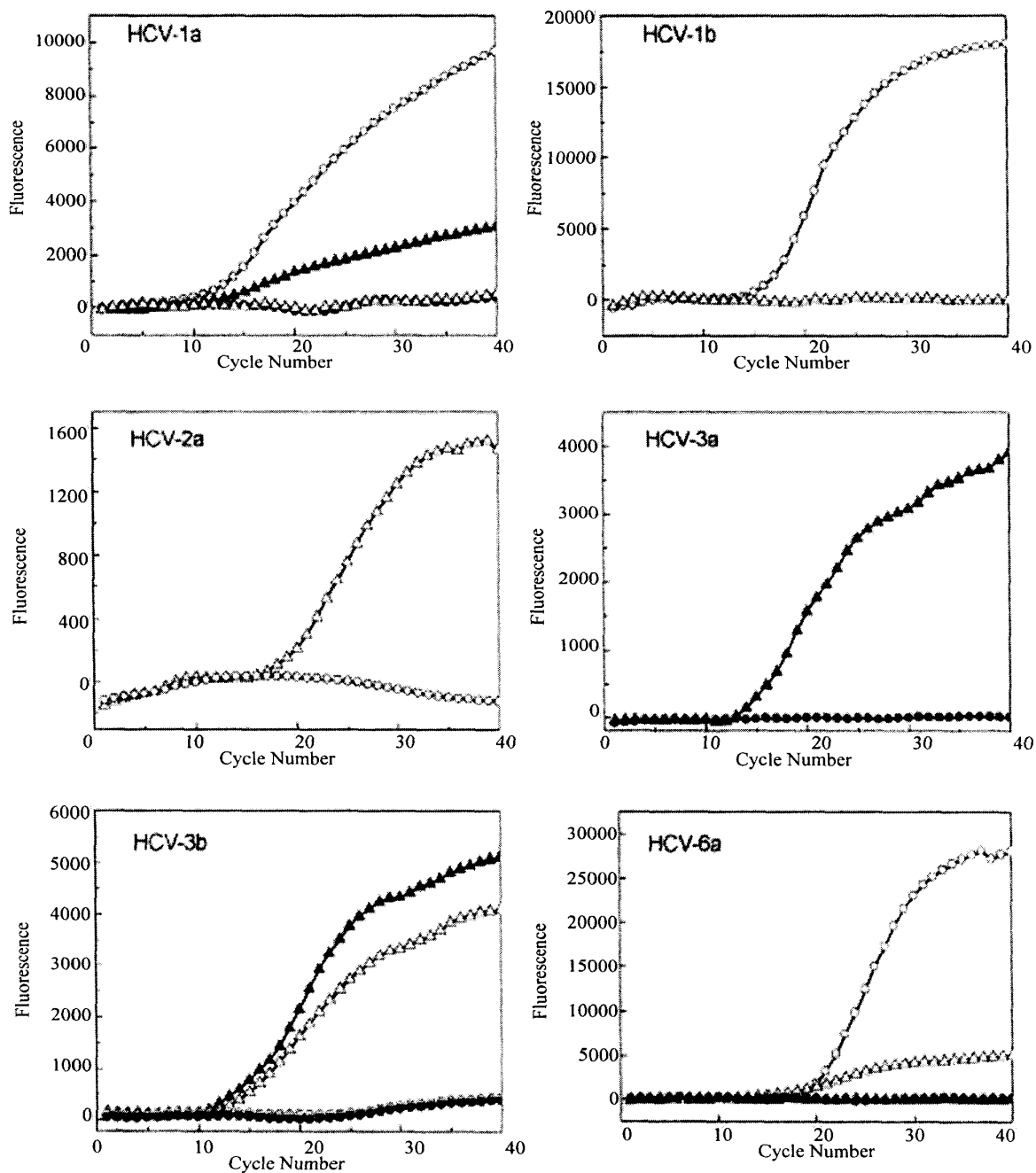


图 4



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(54) **Improved methods for nucleic acid amplification.**

(57) Methods are provided for enhanced specificity and sensitivity of nucleic acid amplification. The methods are simplified nested amplification procedures wherein both inner and outer primer pairs are present in the amplification reaction mixture. According to the methods, the thermocycling profile, as well as the sequences, length, and concentration of amplification primers, is modified to regulate which primers are annealed and extended on the target during any particular amplification cycle. The methods described are particularly suitable in PCR amplifications and have numerous applications in molecular biology, medical diagnostics and forensics.

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The present invention provides improved methods and compositions for nucleic acid amplification. The novel methods provided offer enhanced target specificity, and sensitivity over prior methods and are particularly useful for amplification and detection of target sequences by the polymerase chain reaction (PCR). Specifically, according to the present invention, the thermocycling profile as well as the sequence, length, and concentration of amplification primers are modified to provide improved target specificity. The methods of the invention have numerous applications in the fields of molecular biology, medical diagnostics and forensics.

In one aspect, the present invention provides a method for nested amplification of a sequence within a target nucleic acid in a sample, the method comprises: (a) mixing the sample in an amplification reaction mixture containing an outer primer pair and an inner primer pair, wherein the outer primer pair is capable of amplifying a segment of the target nucleic acid to provide an amplified target sequence, and the inner primer pair is capable of amplifying a subsequence within the target sequence; (b) treating the amplification reaction mixture of step (a) in an amplification reaction at a temperature for annealing and extending the outer primer pair on the target nucleic acid and at a temperature for denaturing the extension products of the outer primer pair to provide an amplified target sequence, wherein the temperature for annealing and extending the outer primer pair is higher than the temperature for annealing and extending the inner primer pair to the target nucleic acid; and (c) treating the mixture of step (b) in an amplification reaction at a temperature for annealing and extending the inner primer pair on the amplified target sequence, and at a temperature for denaturing the extension products of the inner primer pair to provide an amplified subsequence.

In another embodiment of the method, at step (c), the temperature for denaturing the extension products of the inner primer pair is suitable for denaturing the extension products of the outer primer pair and the method further comprises: (d) amplifying the subsequence, at a temperature for annealing and extending the inner primer pair on the amplified target sequence, and at a temperature for denaturing only the extension products of the inner primer pair to provide an amplified subsequence, wherein the temperature for denaturing only the extension products of the inner primer pair is lower than the temperature for denaturing the extension products of the outer primer pair.

In another aspect, the invention provides a method of improved specificity in a PCR that comprises: (a) mixing a sample containing a target nucleic acid sequence in an amplification reaction mixture containing a primer pair for specifically amplifying the target; (b) amplifying the target sequence at a temperature for annealing and extending the primer pair that is between 1°C and 10°C higher than the T_m for the primer pair, and (c) amplifying the amplification reaction mixture of step (b) at a temperature for efficiently annealing and extending the primer pair to provide an amplified target sequence.

The disclosed nucleic acid amplification methods and compositions offer the advantages of enhanced specificity and sensitivity over prior methods for amplifying nucleic acids. The methods relate to the use of modified thermocycling procedures for achieving improved amplification results. In one aspect of the invention, the methods also relate to the use of nested primers for increased specificity. The invention eliminates the disadvantages of prior nested priming procedures and reduces amplification artifacts such as primer-dimer. The improved amplification methods also enhance detection by increasing the amount of target-specific amplified product.

Methods for nucleic acid detection are generally accomplished using oligonucleotide probes. For example, the U.S. Patent Specification No. 4,358,535 discloses a method for detecting pathogens by spotting a sample (e.g., blood, cells, saliva, etc.) on a filter, lysing the cells, and fixing the DNA through chemical denaturation and heating. Then labelled DNA probes are added and allowed to hybridize with the fixed sample DNA. Hybridization indicates the presence of the pathogen's DNA.

The sensitivity and specificity of nucleic acid detection methods was greatly improved by the invention of the polymerase chain reaction (PCR). PCR is a process for amplifying nucleic acids and involves the use of two oligonucleotide primers, an agent for polymerization, a target nucleic acid template, and successive cycles of denaturation of nucleic acid and annealing and extension of the primers to produce a large number of copies of a particular nucleic acid segment. With this method, segments of single copy genomic DNA can be amplified more than 10 million fold with very high specificity and fidelity. PCR methods are disclosed in the U.S. Patent Specification No. 4,683,202 and methods for detecting PCR products are particularly described in the U.S. Patent Specification No. 4,683,195.

Sensitivity is critical when the amplification target is a rare sequence. The detection sensitivity is limited by the amount of amplified product available to be detected. Rare sequences, for example, AIDS virus nucleic acids in an AIDS positive but otherwise healthy individual, or a rare gene transcript indicative of oncogenesis, are very difficult to detect and can be overlooked in conventional analytical practices. Forensic samples often comprise minute amounts of nucleic acid or partially degraded DNA.

A difficulty in detecting a rare sequence is that there can be a high ratio of non-target to target sequence. The ability of a PCR to discriminate between target and nontarget DNA and amplify only target sequences is a key aspect of improved sensitivity. Discrimination between non-target and target is a reflection of the specificity of the primers and reaction conditions. The more specific a reaction is the greater the relative amount of the specific target sequence that is produced and the easier that product is to detect. An increase in specificity can, therefore, increase sensitivity as well.

The need for improved sensitivity and specificity is addressed in the above U.S. Patent Specification No. 4,683,195, which describes the use of nested primers for increasing PCR sensitivity in the amplification of single copy genes. The procedure requires that following PCR the reaction mixture is diluted 10-fold to reduce the concentration of the first primer pair, and a second primer pair is introduced into the reaction mixture and PCR thermocycling is repeated. According to the method, the second primer pair is designed to be internal to the first primer pair to amplify a subsegment of the first PCR product. The method increases specific amplification, i.e., reduces non-specific background amplification products and therefore increases sensitivity. Such non-specific amplification products, although they arise by virtue of fortuitous partial homology to the flanking primers, are unlikely to also have sufficient homology to the nested primers to continue to amplify.

The two step reaction has several drawbacks resulting from the presence of the first primer pair in the second PCR, and the need for additional reagents, including enzyme, following the dilution of the first PCR mixture. The drawbacks of the nested priming method are compounded by the potential for cross contamination between samples during the dilution steps. Due to the enormous amplification possible with the PCR process, small levels of DNA carryover from samples with high DNA levels, positive control templates or targets from previous amplifications can result in PCR product, even in the absence of purposefully added template DNA. Particular methods and precautions for practicing PCR with a minimum of cross contamination are described in Nature 339, 237-238 (1989) and PCR Protocols: A Guide to Methods and Applications, Academic Press, Inc., San Diego, California (1990).

In general, alternative procedures for enhanced specificity involve modified product detection procedures. For example, Nature 324, 163-166 (1986), describes a method for detecting allelic sequence variations due to single-base substitutions in human genomic DNA. The publication provides allele-specific oligonucleotide (ASO) probes that will only anneal to sequences that perfectly match the probe; a single mismatch being sufficient to prevent hybridization. The ASO probes are used in conjunction with amplification of a target segment containing an allelic sequence of interest.

Modifications to the nested primer method mentioned above have been described. Proc. Natl. Acad. Sci. USA 86, 2423-2437 (1989) describes a modified nested priming procedure wherein the internal primers are labeled for subsequent capture and detection. Proc. Natl. Acad. Sci. USA 87, 4580-4584 (1990) describes a "heminesting method" for allele-specific detection. The method requires amplification using a generic primer pair followed by a 1:50 dilution of the PCR product. The product is then re-amplified using three nested, e.g., two upstream and one downstream, primers. For any particular allele only one nested primer is sufficiently complementary to the target to be extended. The primers differ in length and the size of the PCR product allows the allelic state to be determined.

Proc. Natl. Acad. Sci. USA 86, 2757-2760 (1989) describes a method for allele-specific amplification for diagnosing of sickle cell anemia. According to the method, three primers are included in the amplification reaction: two allele-specific primers, one specific for the sickle cell allele and one specific for the normal allele, and a third primer for amplifying either allele. Under stringent conditions, the allele-specific primer will only be extended on the complementary target. Accordingly, only one primer pair will function in PCR for any particular target. Proc. Natl. Acad. Sci. USA 86, 6215-6219 (1989) also describes allele-specific amplification. In a non-nested procedure, primer sequences were modified to distinguish several particular alleles by selective amplification.

Thus, there is a need for simplified methods for increasing both the specificity and sensitivity of nested primer amplification. Improved methods are desirable that also eliminate processing steps and minimize the opportunity for cross contamination and subsequent inaccurate results. The present invention meets those needs.

Brief Description of the Drawings:

Figure 1 provides a schematic description of "Drop-In" PCR;
 Figure 2 provides a schematic description of the "Drop-In/Drop-Out" method demonstrated at Example I;
 Figure 3 provides the results of PCR amplification using the Drop-In/Drop-Out method for detecting HIV nucleic acids; the experiment is described in Example I;

Figure 4 provides the results of the Drop-in/Drop-out PCR method to detect a single copy of HIV DNA in a sample as described in Example I; and

Figure 5 demonstrates the increased specificity provided by the present methods and is described in Example II.

5 The present invention provides improved methods for nested primer amplification. The methods offer improved specificity and sensitivity over prior methods. According to the invention, the thermocycling conditions of the amplification reaction are modified to provide improved results. The sequence, length, and/or concentration of amplification primers are modified as well. In addition, in one embodiment only two primers are required when the temperature cycling conditions are modified during amplification. Generally, 10 however, the methods provide that more than two amplification primers are included in the amplification reaction mixture. The methods of the invention have fewer steps than prior nested amplification methods and consequently offer speed and simplicity over prior nested priming methods.

The methods are particularly suitable in PCR-based amplification techniques wherein a first and a second primer are present in the reaction for amplifying a target nucleic acid. According to the invention, for 15 improved nested amplification, a third primer capable of hybridizing to the amplification product generated by the first and second primers is included in the amplification reaction. The modified nested priming methods utilize the distinct properties of each primer in the amplification reaction mixture to control which primers are extended during each thermocycle. The methods are suitable for amplification of any particular target sequences. In a clinical setting, the methods offer speed, simplicity and decreased opportunity for 20 cross-contamination between samples by minimizing the number of manipulative steps of prior nested amplification methods.

The present methods are suitable for use in any nucleic acid amplification process that is characterized by primer extension and thermocycling. The embodiments of the invention provided herein are PCR-based methods, although the invention is not limited to PCR amplification. PCR is widely practiced in the arts that 25 relate to the present invention and a brief summary of the generic PCR process is provided for convenience.

Amplification of DNA by PCR is disclosed in the U.S. Patent Specifications Nos. 4,683,195 and 4,683,202. Methods for amplifying and detecting nucleic acids by PCR using a thermostable enzyme are disclosed in the U.S. Patent Specification No. 4,965,188.

30 PCR amplification of DNA involves repeated cycles of heat-denaturing the DNA, annealing two oligonucleotide primers to sequences that flank the DNA segment to be amplified and extending the annealed primers with DNA polymerase. The primers hybridize to opposite strands of the target sequence and are oriented so that DNA synthesis by the polymerase proceeds across the region between the primers, effectively doubling the amount of the DNA segment. Moreover, because the extension products 35 are also complementary to and capable of binding primers, each successive cycle essentially doubles the amount of DNA synthesized in the previous cycle. This results in the exponential accumulation of the specific target fragment, at a rate of approximately 2^n per cycle, where "n" is the number of cycles.

The term "amplification reaction system" refers to any in vitro means for multiplying the copies of a target sequence of nucleic acid. Such methods include but are not limited to polymerase (PCR), DNA 40 ligase, (LCR), Q β RNA replicase and RNA transcription-based (TAS and 3SR) amplification systems.

The term "amplifying" which typically refers to an "exponential" increase in target nucleic acid is being used here in to describe both linear and exponential increases in the numbers of a select target sequence of nucleic acid.

The term "amplification reaction mixture" refers to an aqueous solution comprising the various reagents 45 used to amplify a target nucleic acid. These include enzymes, aqueous buffers, salts, amplification primers, target nucleic acid and nucleoside triphosphates. Depending upon the context, the mixture can be either a complete or incomplete amplification reaction mixture. The systems described herein are practiced routinely by those of skill in the relevant art. They have been described in detail by others.

This invention is not limited to any particular amplification system. As other systems are developed, those systems may benefit by practice of this invention. A recent survey of amplification systems was 50 published in Bio/Technology 8, 290-293 (1990). In addition to PCR, the ligase chain reaction (LCR) is an alternative thermocycling amplification procedure. A combined PCR/LCR procedure is suitable for use in conjunction with the present invention. A brief summary of the ligase chain reaction is provided below for the convenience of those not familiar with ligase based amplification systems and to provide an understanding 55 of the breadth of the present invention.

LCR is described in PCT Patent Publication No. WO 89/09835. The process involves the use of ligase to join oligonucleotide segments that anneal to the target nucleic acid. LCR results in amplification of an original target molecule and can provide millions of copies of product DNA. Consequently, the LCR results

in a net increase in double-stranded DNA. The present detection methods are applicable to LCR as well as PCR. LCR requires an oligonucleotide probe for detecting the product DNA.

In the disclosed embodiment, Taq DNA polymerase is preferred although this is not an essential aspect of the invention. Taq polymerase, a thermostable polymerase, is active at high temperatures. Methods for the preparation of Taq are disclosed in the U.S. Patent Specification No. 4,889,818. Taq polymerase is commercially available as a recombinant product or purified from *Thermus aquaticus*. However, other thermostable DNA polymerases isolated from *Thermus* species or non *Thermus* species (e.g., *Thermus thermophilus* or *Thermotoga maritima*), as well as non-thermostable DNA polymerase such as T4 DNA polymerase, T7 DNA polymerase, *E. coli* DNA polymerase I, or the Klenow fragment of *E. coli*, can also be used in PCR. The nucleoside-5'-triphosphates utilized in the extension process, typically dATP, dCTP, dGTP and dTTP, are present in total concentration typically ranging from 400 μ M to 4.0 mM during the extension reaction, although preferably the concentration is between 500 μ M and 1.5 μ M.

Taq polymerase can be prepared as both a 94 kDa and a 62 kDa enzyme. The 62 kDa enzyme is a processed form of the 94 kDa enzyme resulting from proteolytic cleavage of the N-terminal region. Either form of the enzyme will function as an agent of polymerization in PCR. In addition to the N-terminal deletion, individual amino acid residues may be modified by oxidation, reduction or other derivatization, or the protein may be cleaved to obtain fragments that retain activity.

Thus, modifications to the primary structure itself by deletion, addition or alteration of the amino acids incorporated into the sequence during translation can be made without destroying the high temperature DNA polymerase activity of the protein. Such substitutions or other alterations result in proteins useful in the methods of the present invention.

In one aspect of the present invention, a thermostable DNA polymerase that lacks 5' to 3' exonuclease activity is suitable. During extension of an outer primer, an inner primer, complementary to the same template strand, momentarily annealing to its target, is possibly degraded by the 5' to 3' exonuclease of activity of the wild-type DNA polymerase. The net effect of such degradation would be a decrease in the concentration of the inner primer. According to the invention, the inner primer is present in excess; therefore, the use of an enzyme lacking 5' to 3' exonuclease activity is not essential for practicing the present invention. However, using a thermostable DNA polymerase with no or reduced 5' to 3' exonuclease activity provides a preferred form of practicing the disclosed methods of amplification. Such polymerase are known and commercially available.

The term "oligonucleotide" as used herein is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three, and usually more than ten. The exact size will depend on many factors, which in turn depends on the ultimate function or use of the oligonucleotide. The oligonucleotide may be derived synthetically or by cloning.

The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is initiated, i.e., in the presence of different nucleoside triphosphates and a DNA polymerase in an appropriate buffer ("buffer" includes pH, ionic strength, cofactors, etc.) and at a suitable temperature.

The term "thermocycling profile" as used herein refers to the selected temperature parameters selected for "n" cycles of PCR. The thermocycling profile includes at least two temperatures, a high denaturation temperature, adequate for sample-template and subsequent product denaturation and a low temperature appropriate for primer annealing and polymerase extension. Accordingly, in the present invention, particular thermocycling parameters are selected to control primer annealing and product denaturation and thus regulate template accessibility and primer extension.

The choice of primers for use in PCR determines the specificity of the amplification reaction. Primers used in the present invention are generally oligonucleotides, usually deoxyribonucleotides several nucleotides in length, that can be extended in a template-specific manner by the polymerase chain reaction. The primer is sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization and typically contains 10-30 nucleotides, although the exact number is not critical to the successful application of the method.

In one aspect of the invention, short primers which generally require cooler temperatures to form sufficiently stable hybrid complexes with the template are provided to facilitate, via manipulation of the annealing temperature during thermocycling, the beginning of the nested primer phase of the amplification which ultimately enhances sensitivity and specificity of the amplification reaction. As used herein, short primers are characterized as less thermally stable when annealed to target DNA than flanking outer primers. Accordingly, short primers are generally 8-18. In another aspect of the invention, long primers containing a

non-complementary tail, adding 20-100 nucleotides to the 10-30 nucleotide homologous primer region, are provided. The non-complementary regions are useful for providing extension products of increased thermostability which require higher temperatures for subsequent denaturation to serve as amplification templates.

5 Synthetic oligonucleotides can be prepared using the triester method described in J. Am. Chem. Soc. 103, 3185-3191 (1981). Alternatively automated synthesis may be preferred, for example, on a DNA Synthesizer using cyanoethyl phosphoramidite chemistry.

A primer is selected to be "substantially" complementary to a strand of the template having a specific sequence. For primer extension to occur, the primer must be sufficiently complementary to anneal to the
10 nucleic acid template under the reaction conditions. Not every nucleotide of the primer must anneal to the template for primer extension to occur. The primer sequence need not reflect the exact sequence of the template. For example, in one embodiment of the invention, a non-complementary nucleotide fragment or tail is attached to the 5' end of the primer with the remainder of the primer sequence being complementary to the template. Alternatively, non-complementary bases can be interspersed into the primer, provided that
15 the primer sequence has sufficient complementarity with the template for hybridization to occur and allow synthesis of complementary DNA strand.

The choice of the specific nucleic acid target region to be amplified dictates primer sequence and amplification specificity. For example, primers to conserved regions may amplify a class of gene sequences, while primers complementary to variable regions confer greater product specificity.

20 The present invention is particularly suitable for allele-specific amplification. In one embodiment of the invention, the outer primers function as generic primers, and the inner primer, or primers, are allele-specific. According to the present method, the outer primers generate a PCR product which then serves as a template to generate a second smaller PCR product. Thus, the detection of the larger, generic PCR product, specified by the outer primers, provides an internal positive control. The allele-specific amplifica-
25 tion product is generated in the same reaction vessel without opening the tube. This advantage is also particularly significant in clinical screening where thousands of samples are simultaneously screened for the presence of a rare agent, such as AIDS virus nucleic acids or in forensic analyses where a sample is archival or provides evidence of a crime and the amount of sample is extremely limited.

Amplification systems such as PCR require a target nucleic acid in a buffer compatible with the
30 enzymes used to amplify the target. The target nucleic acid can be isolated from any source of biological materials including tissues, body fluids, feces, sputum, saliva, plant cells, bacterial cultures, and the like.

To amplify a target nucleic acid sequence in a sample, the sequence must be accessible to the components of the amplification system. In general, this accessibility is ensured by isolating the nucleic acids from a crude biological sample. A variety of techniques for extracting nucleic acids from biological
35 samples are known in the art. For example, see those described in *Molecular Cloning: A Laboratory Manual* (New York, Cold Spring Harbor Laboratory, 1982), *Nucleic Acid Hybridization: A Practical Approach* (Eds. Hames and Higgins, IRL Press, 1985) or in *PCR Protocols*, Chapters 18-20 (Innis et al., Ed., Academic Press, 1990).

In general, the nucleic acid in the sample will be a sequence of DNA, most usually genomic DNA.
40 However, the present invention can also be practiced with other nucleic acids, such as messenger RNA, ribosomal RNA, viral RNA or cloned DNA. Suitable nucleic acid samples include single or double-stranded DNA or RNA for use in the present invention. Those of skill in the art will recognize that whatever the nature of the nucleic acid, the nucleic acid can be amplified merely by making appropriate and well recognized modifications to the method being used.

Those skilled in the art will know that the PCR process is most usually carried out as an automated
45 process with a thermostable enzyme. In this process, the reaction mixture is cycled through a denaturing temperature range, a primer annealing temperature range, and an extension temperature range. Generally, the annealing and extension temperature ranges overlap, and consequently, PCR is often practiced as a two step cycling reaction comprising a denaturing step and an annealing/extension step. A machine specifically
50 adapted for use with a thermostable enzyme is disclosed more completely in EP Patent Publication No. 236,069 and is commercially available.

In the process described herein, a sample is provided which contains, or is suspected of containing, a particular oligonucleotide sequence of interest, the "target nucleic acid". The target may be RNA or DNA or
55 an RNA/DNA hybrid. The target may be single-stranded or double-stranded. Target preparation will be carried out in a manner appropriate for the particular amplification process to be implemented. For example, in a PCR method where the target nucleic acid is single-stranded, such as mRNA, the target may be first reverse transcribed into cDNA, prior to amplification.

Detection of the amplified products can be accomplished by a number of known means. Such means

include, but are not limited to, hybridization with isotopic or non-isotopically labeled probes in, for example, a dot blot or electrophoretic format. A detection format system may include a capture step, such as a solid support substrate and an avidin-biotin label system. EP Patent Publication No. 237,362 describes a PCR-based detection method termed "reverse" dot-blot in which the probe, instead of the amplified DNA, is fixed to the membrane. According to the method, the target, rather than the probe, is labeled for hybridization.

There are a number of ways to determine whether a probe has hybridized to a DNA sequence contained in a sample. Typically, the probe is labeled in a detectable manner. The target DNA (i.e., the amplified DNA in the PCR-reaction buffer) is bound to a solid support, and determination of whether hybridization has occurred involves determining whether the label is present on the solid support. This procedure can be varied, however, and is possible when the target is labeled and the probe is bound to the solid support.

Many methods for labeling nucleic acids, whether probe or target, are known in the art and are suitable for purposes of the present invention. Suitable labels may provide signals detectable by fluorescence, radioactivity, colorimetry, X-ray diffraction or absorption, magnetism, enzymatic activity, and the like. Suitable labels include fluorophores, chromophores, radioactive isotopes (particularly ^{32}P and ^{125}I), electron-dense reagents, enzymes and ligands having specific binding partners. Enzymes are typically detected by their activity. For example, horse-radish-peroxidase (HRP) can be detected by its ability to convert diaminobenzidine to a blue pigment. A preferred method for HRP-based detection uses tetramethylbenzidine (TMB).

In a homogeneous assay system requiring that amplification occurs in the presence of a detectable DNA binding agent, for example, ethidium bromide, the fluorescence of the amplification mixture increases as the target is amplified and the amount of double-stranded DNA present in the reaction mixture increases. The present invention is particularly suitable for use in conjunction with such a homogeneous assay method. The combined method is described in Example II and provides tremendous advantages over prior methods for amplifying and detecting nucleic acids.

The homogeneous assay method offers means for detecting the presence of target without opening the reaction vessel once the reaction is initiated. The present invention provides a method for nested amplification, also without opening the reaction vessel. Thus, according to the present invention, the combined method has the enhanced sensitivity and specificity of nested priming and the ease of detection of the homogeneous assay system. Without opening the reaction vessel, a sequence as rare as one copy in 70,000 cells can be detected without the additional processing or manipulative steps usually required for target detection. The possibility of cross contamination is greatly minimized by the invention. In another embodiment, the use of a spectrophotometer allows quantitation of the amount of product generated. Consequently, when used in a homogeneous assay, the present invention permits quantitative analysis for monitoring the progress of, for example, an infection or response to treatment regimen.

In general, it is preferred but not essential that the DNA polymerase is added to the PCR reaction mixture after both the primer and template are added. Alternatively, for example, the enzyme and primer are added last or the PCR buffer or template plus buffer are added last. It is generally desirable that at least one component that is essential for polymerization not be present until such time as the primer and template are both present, and the enzyme can bind to and extend the desired primer/template substrate. This method, termed "hot start," improves specificity and minimizes the formation of "primer-dimer."

"Primer-dimer" refers to a double-stranded PCR product consisting of two PCR primers and their complementary sequences. The synthesis of primer-dimer results from the extension of one primer using the other primer as template. Primer-dimer can occur in the absence of target and appears to be a function of primer concentration. The initial formation of "primer-dimer" can occur at low temperatures (i.e., room temperature); "hot start" eliminates this phase of the thermal profile. Once formed, this PCR artifact is amplified very efficiently and is detected in reactions with rare, or no specific template. In rare template reactions, the primer-dimer can compete with the target fragment for primers and enzyme and prevent efficient target amplification.

The use of nested primers serves to decrease primer-dimer and enhance PCR specificity. U.S. Patent Specification No. 4,683,195 describes that the nested primer procedure requires two primer pairs. According to the method a first "outer" primer pair is used to amplify a target sequence and a second "inner" primer pair is used to amplify a subsegment of the PCR product formed from the first amplification reaction. According to the method described therein, the reaction mixture is diluted 10-fold to reduce the concentration of the first primer pair after PCR, then the second primer pair is introduced into the reaction mixture. This method requires a number of additional steps, including, stopping the PCR, opening the reaction tube, diluting the reaction to eliminate or decrease the concentration of outer primers, adding a second primer pair, consequently adjusting buffer conditions, providing additional enzyme, and a second thermocycling

reaction. These steps are eliminated by the present invention.

The present invention provides nested amplification methods and compositions wherein the outer and inner primers are all present in the initial reaction mixture, and the thermocycler is programmed to allow the outer primers, but not the inner primers, to amplify initially, and then to allow the inner primers, but not the outer primers, to amplify the targeted subsequence within the initial PCR product. The examples of the invention serve to illustrate that primers prepared according to the invention are made to "drop in" or "drop out" of the amplification reaction in accord with the desired result. In several embodiments, one member of the initial flanking i.e., outer, primer pair also functions as an inner or secondary primer. Consequently, only three primers are utilized. Alternatively, four individual primers are used, comprising both inner and outer primer pairs. When four primers are employed, either one or both of the inner primers are made to "drop-in."

According to the present invention, a third primer, internal to one member of the outer primers is included in a PCR reaction for amplifying a particular target segment. As the temperature in the PCR reaction is raised, a point is reached at which this third primer no longer stably anneals to the target template, while the flanking primers continue to stably anneal. Thus, the third primer has an optimum, annealing temperature for PCR, that is lower than the optimum annealing temperature of the flanking outer primer. This property can be imparted to the third primer by virtue of a shorter length and/or lower G-C content. Adjusting the annealing temperature during the amplification reaction determines which primer pair is extended by the polymerase during any particular annealing/extension cycle.

In one embodiment of the invention, the primer flanking the third primer is present at a low concentration. For the first n PCR cycles, the temperature during the extension phase of each PCR cycle is maintained sufficiently high, e.g., approximately 65 °C to prevent the short, inner primer(s) from annealing specifically and initiating amplification. The outer primers anneal sufficiently such that PCR proceeds normally at the high extension temperature. Prior to amplification plateau, when the supply of limiting outer primer is almost exhausted, the annealing temperature is decreased, for example, to approximately 42 °C. At this temperature, the third primer "drops in" and proceeds to amplify the target for the remaining cycles. This procedure is schematically described in Figure 1 and referred to herein as "drop-in" PCR. According to the figure, the outer primer pair comprises primers "A" and "C," the inner primer pair comprises primers "B" and "C," and primer "B" is the third primer.

DNA and Cell Biology 10, 233-238 (1991) provides a formula for estimating the optimum annealing temperature for PCR for any particular primer/template duplex. The nearest neighbour thermodynamic data disclosed in Proc. Natl. Acad. Sci. 83, 3746-3750 (1986), as well as methods described in Nuc. Acids Res. 6, 3543-3557 (1979) exemplify empirical formula for estimating T_m . Although T_m is generally slightly higher than the optimum annealing temperature for PCR, calculating T_m provides a guidance for one of ordinary skill in the art to empirically determine suitable temperatures for practicing the present invention.

For some targets, for example, HIV proviral DNA in a fixed amount of peripheral blood from HIV infected individuals, the amount of target present in a sample varies up to 1,000-fold. Drop-in PCR is particularly suitable for such an analysis because of the effect of limited outer primer concentration on the amplifications. In a particular sample, if the amount of target is high, the PCR reaches plateau quickly but not completely. If the amount of target in a sample is low, amplification continues. Consequently, using a limiting outer primer concentration, different reactions are brought to about the same state of limited amplification before the thermocycling conditions are altered to allow the nested primer to "drop-in." As a result, the effect of sample to sample variance on nested amplification results is diminished.

In an alternative embodiment for nested primer amplification, the need for a low concentration of one flanking primer is eliminated. According to the method, a high denaturation temperature and a high annealing temperature allow the outer primers, but not the inner primers, to be extended. A flanking primer is synthesized with a G-C rich tail. Because the non-complementary primer tail sequence is incorporated into the PCR product after two cycles of amplification, the G-C tail serves to raise the temperature necessary for denaturing the PCR product because of the increased thermostability of G-C pairs versus A-T pairs. The resulting difference in the denaturing temperature of the nested and flanking PCR products is then exploited to effectively shut down amplification from the tailed outer primer.

Once PCR product is made from the outer primers, and after the nested primers have been allowed to "drop-in," by manipulating the annealing temperature to initiate synthesis from the nested primer when desired, the denaturation temperature is lowered, i.e., from 96 ° to 86 °C. At the lower temperature the outer primer PCR product does not completely denature and consequently cannot serve as a template. However, the lower denaturation temperature is sufficient to allow amplification of the nested PCR product. This aspect of the invention is summarized as follows. After n cycles, the annealing temperature is dropped, allowing the inner primers to anneal. After $n + x$ cycles, the denaturation temperature is dropped,

preventing the initial PCR product from denaturing and serving as a template for the outer primer. This strategy, termed "Drop-in/Drop-out" PCR can be practiced using only one internal primer as described in Examples I and II. In those embodiments, one flanking primer serves as both inner and outer primer and is unaffected by the altered thermal profile of the cycling reaction. Drop-in/Drop-out PCR is schematically described in Figure 2.

One of ordinary skill in the art can readily use empirical means to determine the appropriate denaturation and annealing temperatures for any particular amplification reaction mixture and program a thermocycler accordingly. The drop-in/drop-out PCR method provides means for including high concentrations of all primers which is preferred for optimizing PCR efficiency while controlling which particular primer pair is extended at any cycle during the reaction. This aspect of the method for nested primer amplification is demonstrated in Examples I and II.

The Drop-in PCR method requires that a first outer primer is present at a low concentration. Generally, amplification primers are present at a concentration of 1-100 pmole per 100 μ l reaction. For drop-in PCR a suitable concentration for the limited out primer is within the range of 1% to 50% of the concentration of second and third primers (i.e., the inner primer pair). As amplification proceeds, the amount of first primer becomes limiting, with respect to the other primers. Consequently, when the annealing temperature is decreased to the T_m of the third primer, annealing and extension of the inner primer is favored and the smaller primer PCR product begins to accumulate.

According to the invention, the change in the thermocycling profile during PCR enhances amplification specificity. As PCR proceeds, non-specific primer events lead to a build up of non-target, i.e., background, products. Once a thermocycle, using a non-target template, is complete, that template will continue to be amplified in successive cycles. Although outer primers anneal to the amplified non-target template, the inner primer does not. Each change in the thermocycling profile results in a change in the population of non-target template molecules. Therefore, with each change in the cycling parameters, the non-target amplification possible during the previous thermocycle is diminished, and consequently, target specificity is enhanced.

The number of cycles necessary at each thermocycling profile, or phase, is empirically determined and varies depending on the complexity of the sample, the particular target, and the relative amounts of target and background nucleic acid. The number of cycles required at the high annealing temperature, where in the outer primers are extended, is one, or any number of cycles greater than one. It may be desirable to include, for example, 1-30 cycles at high annealing temperature, and the specific number of cycles is determined according to the particularities of the assay; i.e., sample, target, primers, and the specific result required.

It may be desirable to amplify a first PCR target using outer primers, for a number of cycles sufficient to provide a detectable signal. Using a homogeneous assay the presence of the amplified product is readily determined by fluorescence, without opening the reaction vessel. The outer primers may be suitable for amplifying a region of interest known to be present such as a particular gene segment. Amplification of, for example, a genetic marker by an outer primer pair provides an internal positive control for indicating that amplification has occurred.

Subsequently, the thermocycling profile is altered, and the inner target or specific primers are utilized. The additional increase in PCR product determined by a further increase in fluorescence indicates the presence of a particular target sequence. However, detection of the outer primer pair amplification product is not an essential aspect of the invention. Amplification by the first (outer) primer pair serves to increase the number of target molecules providing additional templates for amplification by the second (inner) primer pair.

The second cycling phase in the Drop-in/Drop-out method (high T denaturation, low T anneal) serves to initiate amplification using the internal primers and provide target for the third cycling phase (low T denaturation, low T anneal). During the third cycling phase, the lowered denaturation temperature effectively eliminates outer primer amplification, while inner primer amplification continues. Amplification at high T denaturation and low T anneal converts outer PCR product to inner PCR template. In practice 1-10 cycles are sufficient for the second phase of the reaction, although more cycles may be desirable for a particular target.

The annealing temperature for any specific primer in PCR is a function of the %GC content of the oligonucleotide. It will be obvious to one of skill in the art to determine the annealing temperature suitable for any particular target and primer using the guidance provided by the present examples of the invention. The difference in the annealing temperature of the low T anneal and high T anneal cycles is at least 1 °C and preferably is 3 ° - 30 °C. Similarly, the primer extension temperature is readily determined by one of ordinary skill in the art using the cited references and the present examples as a guide.

The variation from high T denaturation to low T denaturation is as much as 20 °C or as little as 1 °C depending on the target and sample complexity, G-C content, and primer sequence. The shift to a low denaturation temperature prevents denaturation of non-target amplified DNA. Because this nucleic acid is non-target, the length and %GC content cannot be determined for estimating the optimum temperature for preventing denaturation. Consequently, the denaturation temperature is empirically determined using the present specification and examples as a guide. PCR Technology, Stockton Press (1989) describes the effects of nucleotide sequences on the T_m of DNA subsegments or domains within a larger nucleic acid sequence. For example, a tailed primer comprising approximately 40 nucleotides of 100% GC is sufficient to alter the temperature required for complete denaturation of an amplified sequence 500 base pairs in length. Preferably, the G-C tail, added to the 5' end of the flanking primer is between 10 and 100 nucleotides in length.

In an additional aspect of the invention, a flanking primer is not required for enhanced product specificity. Following the protocol for Drop-in amplification using only the inner primer pair provides an unexpectedly superior result when compared to the results of amplification using standard PCR conditions.

Example II clearly demonstrates this aspect of the invention.

Prior to the present invention, it was not known that amplification specificity is improved by subjecting the sample to at least one thermocycle using an annealing temperature higher than the T_m. The cycling profile is subsequently adjusted and the annealing temperature is lowered to the appropriate T_m. Thus, in one embodiment of the invention the temperature for primer annealing and extension is 1 ° - 10 °C higher than the estimated T_m for the particular primer pair. Although amplification at this temperature is extremely inefficient, any primer extension that occurs is target specific. Consequently, during the high temperature cycle(s), the sample is enriched for the particular target sequence and any number of cycles, i.e., 1-15 enhances product specificity. The annealing temperature is then decreased to increase amplification efficiency and provide a detectable amount of PCR product.

This method is particularly useful for increasing sensitivity as well as specificity. For example, detection of AIDS virus nucleic acids present as 1 copy per 70,000 cells is enhanced by the method and is illustrated in Example I. Similarly, allele-specific amplification for detecting the presence of a one-base pair change is also improved by the addition of the high temperature annealing cycle(s).

In another aspect, the invention may be commercialized providing kits for enhanced amplification specificity. Kits would include inner and outer primer pairs for a specific target or allele. Kits may also include any of the following reagents for amplification such as a DNA polymerase, buffers, dNTPs, and a positive control template.

The examples provided herein offer general guidance for practicing the present invention and are not intended as a limitation to the scope of the invention.

Example I

Drop-In/Drop-Out PCR: Detection of a Rare Target in the Presence of a High Background of Double-Stranded DNA

The drop-in/drop-out amplification method was used for amplifying a rare single copy sequence in a background of DNA from 70,000 human cells. A modified nested primer procedure, as briefly described in the "Detailed Description" section as a primer "drop-in/drop-out" procedure, was designed to enhance PCR specificity. This assay was done as follows: into PCR reaction vessels 1 through 8 were aliquoted 50 microliters of solution, each containing 50 mM KCl; 10 mM TrisHCl, pH 8.3; 2.5 mM MgCl₂; 600 μM total dNTPs; 1.25 unit of Taq DNA polymerase (PECI); 1.27 μM ethidium bromide; 0.5 μg of human cell-line DNA; the primer pair RH171 (SEQ ID NO: 1) and RH176 (SEQ ID NO: 2), each primer at 0.2 μM; and the nested primer RH182 (SEQ ID NO: 3) also at 0.2 μM. A drop of mineral oil was used to cover the eight solutions in order to prevent evaporation. Primer RH176 (SEQ ID NO: 2) carries a GC-rich, non-homologous (to target sequence), 5' "tail" that raises the denaturation temperature necessary to amplify PCR product made using this primer.

Reactions 1-4 were made with solutions that were at ambient room temperature and included the three primers before temperature cycling was begun. Reactions 5-8 were made with the addition of the three primers postponed until these reactions were equilibrated to a temperature of 72 °C before beginning thermocycling. For this reason, reactions 5-8 are referred to as being given a "hot-start." Reactions 2-4 and 6-8 also contained a target, positive control DNA (purchased from PECO) containing HIV sequences to which RH171 (SEQ ID NO: 1), RH182 (SEQ ID NO: 3), and the 3' portion of RH176 (SEQ ID NO: 2) were homologous. This DNA was diluted such that each reaction containing it had, on average, four copies of the