

Table 1: Individual assay linearity and efficiency

Assay	Linearity (R^2)		Efficiency (%)	
	In the absence of 500ng gDNA	In the presence of 500ng gDNA*	In the absence of 500ng gDNA	In the presence of 500ng gDNA
cmEPO	0.992	0.989	95	82
ITC ^{cmEPO}	0.989	0.998	91	86
hEPO	0.999	0.998	92	88
ITC ^{hEPO}	0.996	0.993	89	86
CMV	0.996	1.000	99	97
ITC ^{CMV}	0.997	0.989	98	94

*macaque gDNA with cmEPO and ITC^{cmEPO} assays, and human gDNA with hEPO, ITC^{hEPO}, CMV and ITC^{CMV} assays.

Table 2: Individual assay sensitivity

Assay	Positive Control	Mean* Copy Number (s.e.)	Mean* Ct (s.e.)	CV (%) of Ct
cmEPO	5 copies pDNA with 500ng gDNA (macaque)	6.7 (2.5)	37.24 (0.22)	2.2
	5 copies pDNA with TE	5.9 (3.4)	37.47 (0.28)	2.9
ITC ^{cmEPO}	5 copies ITC template with 500ng gDNA (macaque)	6.5 (2.7)	37.22 (0.24)	1.5
	5 copies ITC template with TE	5.5 (3.8)	37.14 (0.31)	3.2
hEPO	10 copies pDNA with 500ng gDNA (human)	9.6 (3.3)	36.74 (0.14)	1.5
	10 copies pDNA with TE	10.8 (2.8)	36.45 (0.10)	1.1
ITC ^{hEPO}	10 copies ITC template with 500ng gDNA (human)	11.8 (3.9)	36.55 (0.14)	1.6
	10 copies ITC template with TE	11.1 (2.4)	36.73 (0.10)	0.9
CMV	10 copies pDNA with 500ng gDNA (human)	11.9 (5.3)	36.29 (0.18)	2.0
	10 copies pDNA with TE	11.1 (4.1)	36.35 (0.14)	1.5
ITC ^{CMV}	10 copies ITC template with 500ng gDNA (human)	10.7 (4.3)	36.64 (0.16)	1.7
	10 copies ITC template with TE	11.8 (4.0)	36.55 (0.13)	1.4

*From fifteen replicates of plasmid (pDNA) or ITC template. Standard error (in parenthesis).

Table 3: Individual assay specificity

Assay	Negative Control	False positive	False Positive Rate (%)
cmEPO	500ng gDNA (macaque)	0/20	0
ITC ^{cmEPO}	500ng gDNA (macaque)	0/20	0
hEPO	500ng gDNA (human)	0/20	0
ITC ^{hEPO}	500ng gDNA (human)	0/20	0
CMV	500ng gDNA (human)	0/20	0
ITC ^{CMV}	500ng gDNA (human)	0/20	0

Table 4: ITC^{cmEPO} duplex assay testing of WBC samples from NHP transduced IM *in vivo*

rAAV1				
Time Points	Mean [*] cmEPO Ct (s.e.)	Mean ITC ^{cmEPO} Ct ^{**} (s.e.)	Positive/Negative	Actual Copy Number ^{***}
Pre-injection	40.00 [†] (0)	37.00 (0.29)	Negative (P-value <0.0001)	0
3 Days p.i.	32.81 (0.10)	37.21 (0.15)	Positive (P-value <0.0001)	188
7 Days p.i.	35.92 (0.27)	37.48 (0.29)	Positive (P-value =0.0019)	13
14 Days p.i.	36.94 (0.51)	37.46 (0.40)	Positive (P-value =0.4350)	8
10 Weeks p.i.	38.49 (0.49)	37.67 (0.13)	Positive (P-value =0.1066)	2
18 Weeks p.i.	38.32 (0.72)	37.18 (0.73)	Positive (P-value = 0.2913)	3
23 Weeks p.i.	39.91 (0.06)	37.18 (0.23)	Negative (P-value <0.0010)	0

rAAV 8				
Time Points	Mean [*] cmEPO Ct (s.e.)	Mean ITC ^{cmEPO} Ct ^{**} (s.e.)	Positive/Negative	Actual Copy Number ^{***}
Pre-injection	40.00 [†] (0)	37.43 (0.24)	Negative (P-value <0.0001)	0
3 Days p.i.	35.70 (0.49)	36.67 (0.12)	Positive (P-value <0.0875)	10
7 Days p.i.	36.68 (0.35)	36.99 (0.44)	Positive (P-value = 0.5923)	7
14 Days p.i.	37.44 (0.43)	37.43 (0.14)	Positive (P-value = 0.9795)	8
10 Weeks p.i.	40.00 (0)	36.62 (0.33)	Negative (P-value < 0.0001)	0
18 Weeks p.i.	38.02 (0.68)	36.83 (0.68)	Positive (P-value = 0.2528)	2
23 Weeks p.i.	38.17 (0.54)	37.30 (0.19)	Positive (P-value = 0.1432)	3

^{*}From five replicates of each 500ng *in vivo* sample. s.e. = standard error (in parenthesis).

^{**}From an input of five copies of ITC^{cmEPO} template.

^{***}The actual copy number is from our previous study [32].

[†]The number of cycles of the real-time PCR program was set to 40.

Table 5: ITC^{hEPO} duplex assay testing

Copy Number of Spiked Plasmid	Mean [*] hEPO Ct (s.e.)	Mean ITC ^{hEPO} Ct ^{**} (s.e.)	Positive/Negative
0	40.00 [†] (0)	36.93 (0.42)	Negative (P-value <0.0001)
188	32.60 (0.44)	36.48 (0.33)	Positive (P-value <0.0001)
13	36.63 (0.28)	37.12 (0.27)	Positive (P-value =0.2426)
8	36.88 (0.28)	36.84 (0.28)	Positive (P-value =0.9172)
2	39.80 (0.20)	37.32 (0.37)	Negative (P-value <0.0001)

^{*}From five replicates of pShuttle-CAG-hEPO-pA plasmid DNA spiked into 500ng naive human gDNA. Standard error (in parenthesis).

^{**}From an input of 10 copies of ITC^{hEPO} template.

[†]The number of cycles of the real-time PCR program was set to 40.

Table 6: Plasmid spiking of different naive human gDNA samples

Human gDNA	5:10 [*]			10:10 ^{**}			20:10 ^{***}		
	Mean [†] hEPO Ct	Mean ITC ^{hEPO} Ct	P-Value	Mean [†] hEPO Ct	Mean ITC ^{hEPO} Ct	P-Value	Mean [†] hEPO Ct	Mean ITC ^{hEPO} Ct	P-Value
1	37.36 (0.84)	36.25 (0.53)	0.0368	36.80 (0.41)	36.70 (0.83)	0.8853	35.36 (0.46)	36.95 (0.50)	0.0482
2	38.03 (0.40)	36.25 (0.24)	0.0052	36.68 (0.32)	36.99 (0.58)	0.8663	35.17 (0.39)	36.41 (0.14)	0.0168
3	37.45 (0.85)	36.39 (0.38)	0.0253	36.94 (0.44)	37.14 (0.83)	0.7840	35.18 (0.44)	37.46 (0.59)	0.0143
4	37.62 (0.33)	36.24 (0.10)	0.0040	36.91 (0.35)	37.00 (0.79)	0.8924	35.34 (0.45)	36.97 (0.50)	0.0425

Human gDNA	5:10 [*]			10:10 ^{**}			20:10 ^{***}		
	Mean [†] CMV Ct	Mean ITC ^{CMV} Ct	P-Value	Mean [†] CMV Ct	Mean ITC ^{CMV} Ct	P-Value	Mean [†] CMV Ct	Mean ITC ^{CMV} Ct	P-Value
1	36.88 (0.35)	36.27 (0.06)	<0.0001	36.77 (0.08)	36.62 (0.12)	0.3259	36.21 (0.09)	36.65 (0.11)	0.0172
2	36.37 (0.32)	36.70 (0.12)	0.0011	36.56 (0.14)	36.97 (0.15)	0.3333	36.11 (0.11)	36.69 (0.08)	0.0025
3	36.55 (0.42)	36.82 (0.13)	0.0045	36.78 (0.07)	36.74 (0.13)	0.7807	36.23 (0.14)	36.91 (0.13)	0.0081
4	36.83 (0.32)	36.73 (0.18)	0.0005	36.67 (0.12)	36.57 (0.08)	0.9355	36.06 (0.23)	36.71 (0.04)	0.0183

[†]From five replicates of pShuttle-CAG-hEPO-pA plasmid DNA spiked into 500ng naive human gDNA. Standard error (in parenthesis).

[†]From five replicates of pSSV9-MD2-cmEPO plasmid DNA spiked into 500ng naive human gDNA. Standard error (in parenthesis).

^{*}5 copies of plasmid and 10 copies of ITC template.

^{**}10 copies of plasmid and 10 copies of ITC template.

^{***}20 copies of plasmid and 10 copies of ITC template.

[†]The number of cycles of the real-time PCR program was set to 40.

CLAIMS

What is claimed is:

1. A method of detecting exogenous DNA material in a subject, said method comprising:
 - obtaining a DNA containing sample from said subject;
 - conducting real-time PCR on said sample in the presence of a prescribed amount of an ITC template, primers for a target DNA sequence; a target probe that binds to said target DNA sequence and an ITC probe; and
 - determining whether said target DNA sequence amount is equal to or greater than said ITC template amount.
2. The method of claim 1, wherein said target DNA sequence is an exon-exon junction of DNA homologous to an endogenous gene.
3. The method of claim 2, wherein said endogenous gene is erythropoietin (EPO), darbepoetin (dEPO), hypoxia-inducible factor (HIF) stabilizers, Chorionic Gonadotrophin (CG), Luteinizing Hormone (LH); Insulins; Corticotrophins; Growth Hormone (GH), Insulin-like Growth Factor-1 (IGF-1), insulin-like growth factor-2 (IGF2), Fibroblast Growth Factors (FGFs), Hepatocyte Growth Factor (HGF), Mechano Growth Factors (MGFs), myogenin, peroxisome proliferator-activated receptor delta (PPARd), calcineurin-A-alpha, chorionic somatomammo-tropin hormone 1 (CSH1), chorionic somato- mammo-tropin hormone 1/2 (CSH1/CSH2), chorionic somatomammo-tropin hormone 2 (CSH2), chorionic somatomammo-tropin hormone-like 1 (CSHL1), myostatin inhibitor. Platelet-Derived Growth Factor (PDGF), Vascular-Endothelial Growth Factor (VEGF) as well as any other growth factor affecting muscle, tendon or ligament protein

synthesis/degradation, vascularisation, energy utilization, regenerative capacity or fibre type switching; and other substances with similar biological effect(s).

4. The method of claim 1, wherein said ITC template and said target DNA sequence comprise primer binding sequences recognized by said primers.
5. The method of claim 1, wherein the distance between primer binding sequences for the ITC template is the same for the target DNA sequence +/- 50bp.
6. The method of claim 1, wherein said prescribed amount is 3-5 or more copies of said ITC template.
7. The method of claim 6, wherein said prescribed amount is 3-100 copies.
8. The method of claim 6, wherein said prescribed amount is 3-10 copies.
9. The method of claim 1, wherein said subject is a human or non-human mammal.
10. The method of claim 1, wherein said subject is an organism .
11. The method of claim 1, wherein said target DNA sequence is homologous or nonhomologous to the host genome.
12. The method of claim 1, wherein said ITC probe comprises a first marker molecule.
13. The method of claim 12, wherein said first marker molecule is a fluorophore of a first color.
14. The method of claim 1, wherein said target probe comprises a second marker molecule.

15. The method of claim 14, wherein said second marker molecule is a fluorophore of a second color.

16. The method of claim 1, wherein said ITC probe comprises a fluorophore that generates a first color signal and said target probe comprises a fluorophore that generates a second color signal.

17. A system for detecting foreign DNA material in a subject, said system comprising:

a real-time PCR instrument comprising a receptacle for holding a DNA containing sample; and

3 or more copies of an ITC template, primers for a target DNA sequence; target probe specific to said target DNA sequence and an ITC probe disposed in said receptacle.

18. An ITC template comprising a probe binding sequence that binds to a corresponding ITC probe and flanking primer recognizing sequences that binds to primers, wherein said primers also hybridize to primer sites of a separate target DNA sequence, said target DNA sequence lacking the ITC probe binding sequence.

19. The ITC template of claim 18, comprising a first and second primer sequence that flanks said probe binding sequence.

20. A system for detecting presence of a target DNA sequence in a biological sample, said system comprising:

an ITC template comprising a probe binding sequence that binds to a corresponding ITC probe and a first and second flanking primer recognizing sequence that binds to a first and second primer, respectively;

an ITC probe that binds to said probe binding sequence in the ITC template, said ITC probe comprising a first marker molecule;

a first primer that binds to said first flanking primer sequence in the ITC template and binds to sequences in the target DNA;

a second primer that binds to said second primer sequence in the ITC template and binds to sequences in the target DNA; and

a target DNA sequence probe, said target DNA sequence probe comprising a second marker molecule.

21. The system of claim 20, wherein said first marker molecule is a fluorophore of a first color and said second marker molecule is a fluorophore of a second color.

22. The system of claim 20, wherein said first and second marker molecules are different.

23. A kit comprising an ITC template according to claim 18 and an ITC probe.

24. The method according to claim 4, wherein said primers comprise a primer pair a first PCR primer can hybridize at stringent conditions to a first exon on the first strand of the target DNA sequence, and the second PCR primer can hybridize at stringent conditions to a second exon on the strand of the target DNA complementary to said first strand, wherein said second exon is located adjacent to said first exon.

25. The method of claim 2, wherein the target probe hybridizes with a first segment to a first exon and simultaneously with a second segment to a second exon of said target DNA sequence (intron-spanning probe).

26. A method according to claim 25, wherein intron- spanning PCR probe is designed hybridizes to such regions of said first and said second exons on said target DNA, which are conserved among splice variants of such genes from which the coding sequence of the target DNA derives.

27. The method of claim 1, wherein determining comprises (i) determining a Ct of said target probe to obtain a first Ct and (ii) determining a Ct of said ITC probe to obtain a second Ct, wherein when first Ct is equal or lower than said second Ct the sample contains said target DNA sequence.

Figure 1A

ITC Testing for Gene Transfer Vectors
(across transgene cDNA exon-exon junction or on heterologous sequences)

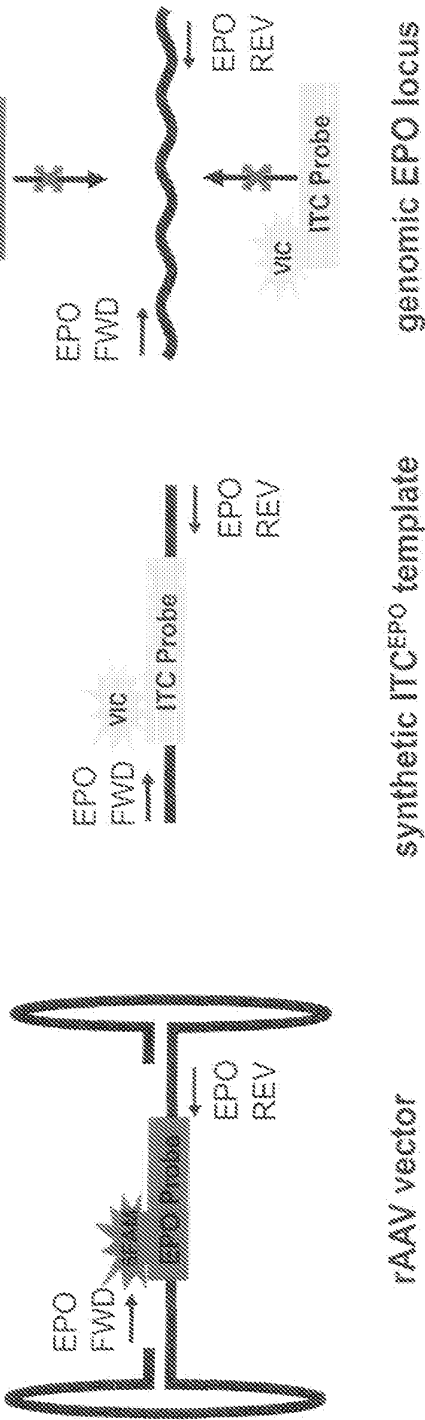


Figure 1B

ITC Testing for Infectious Agents

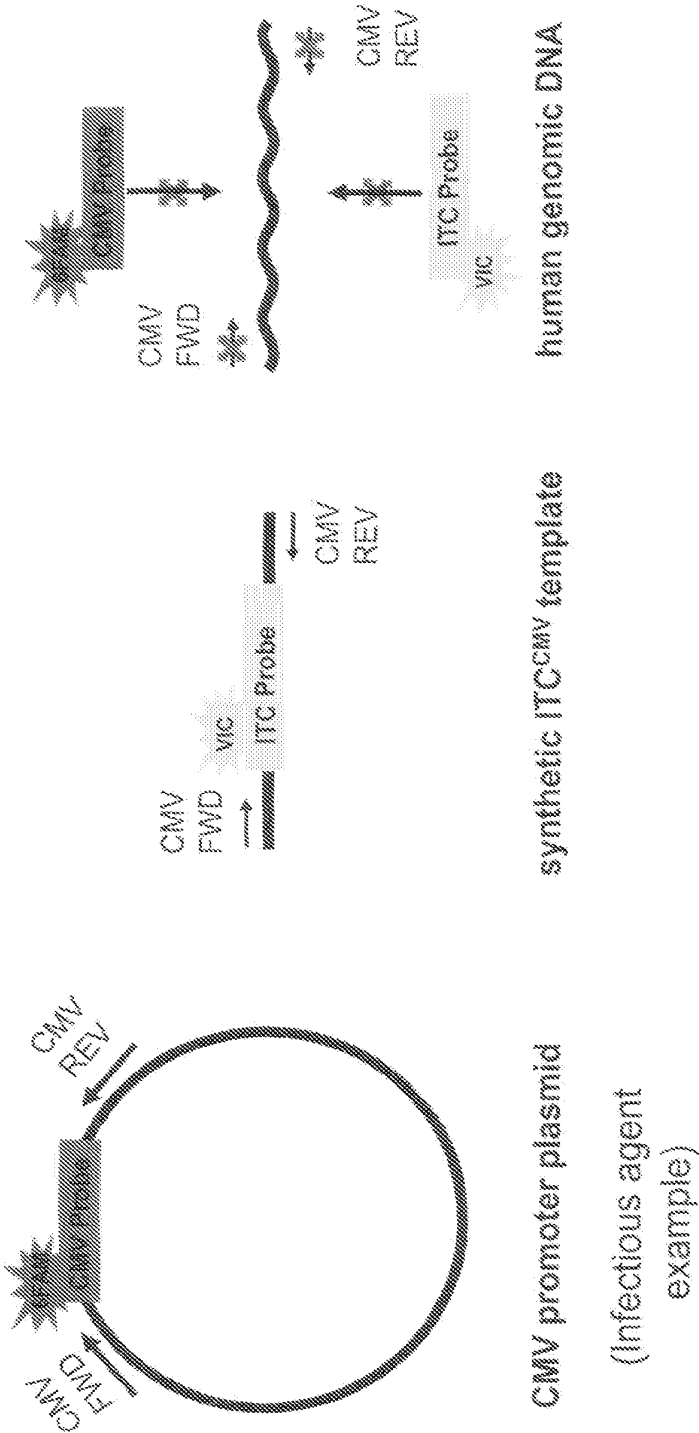
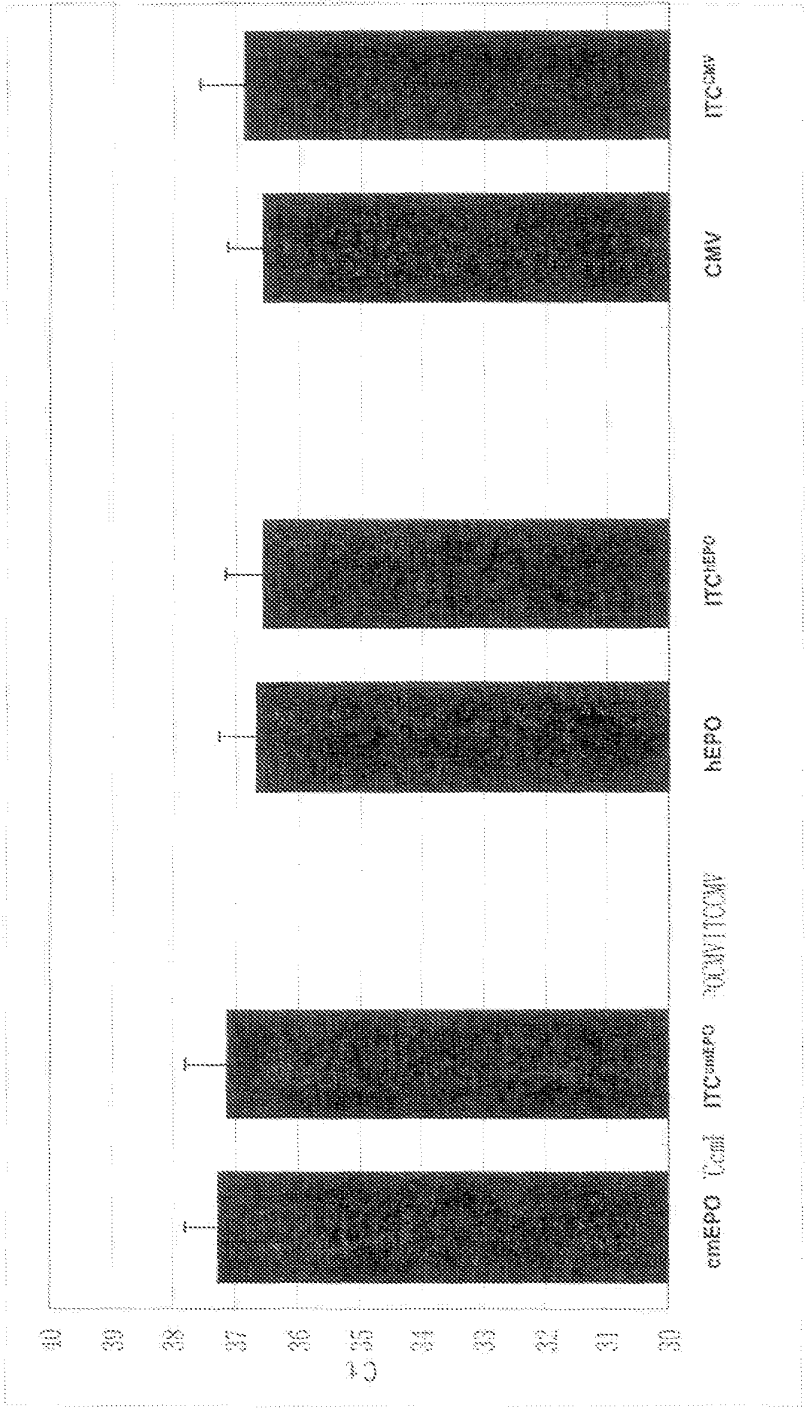


Figure 2



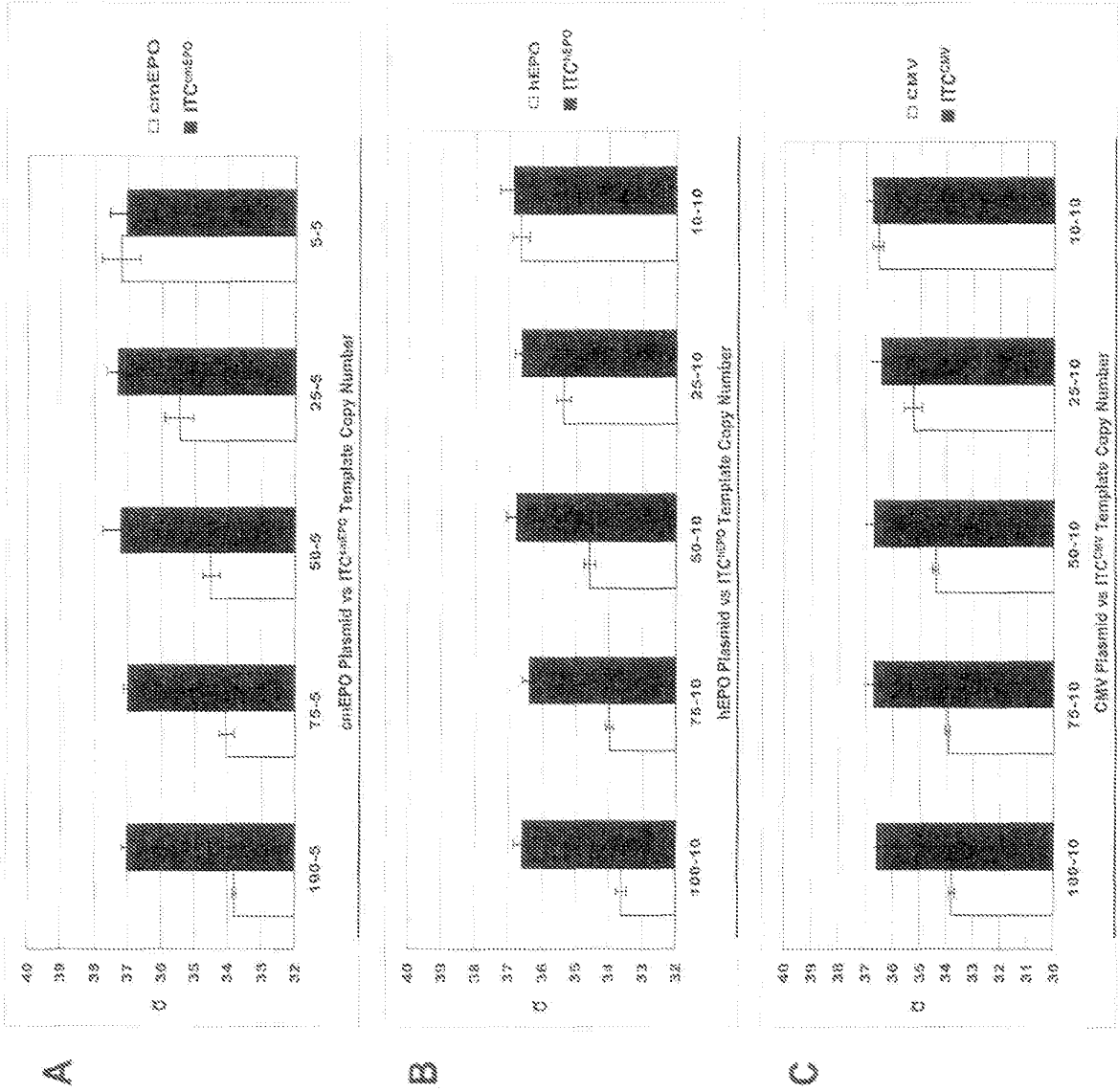
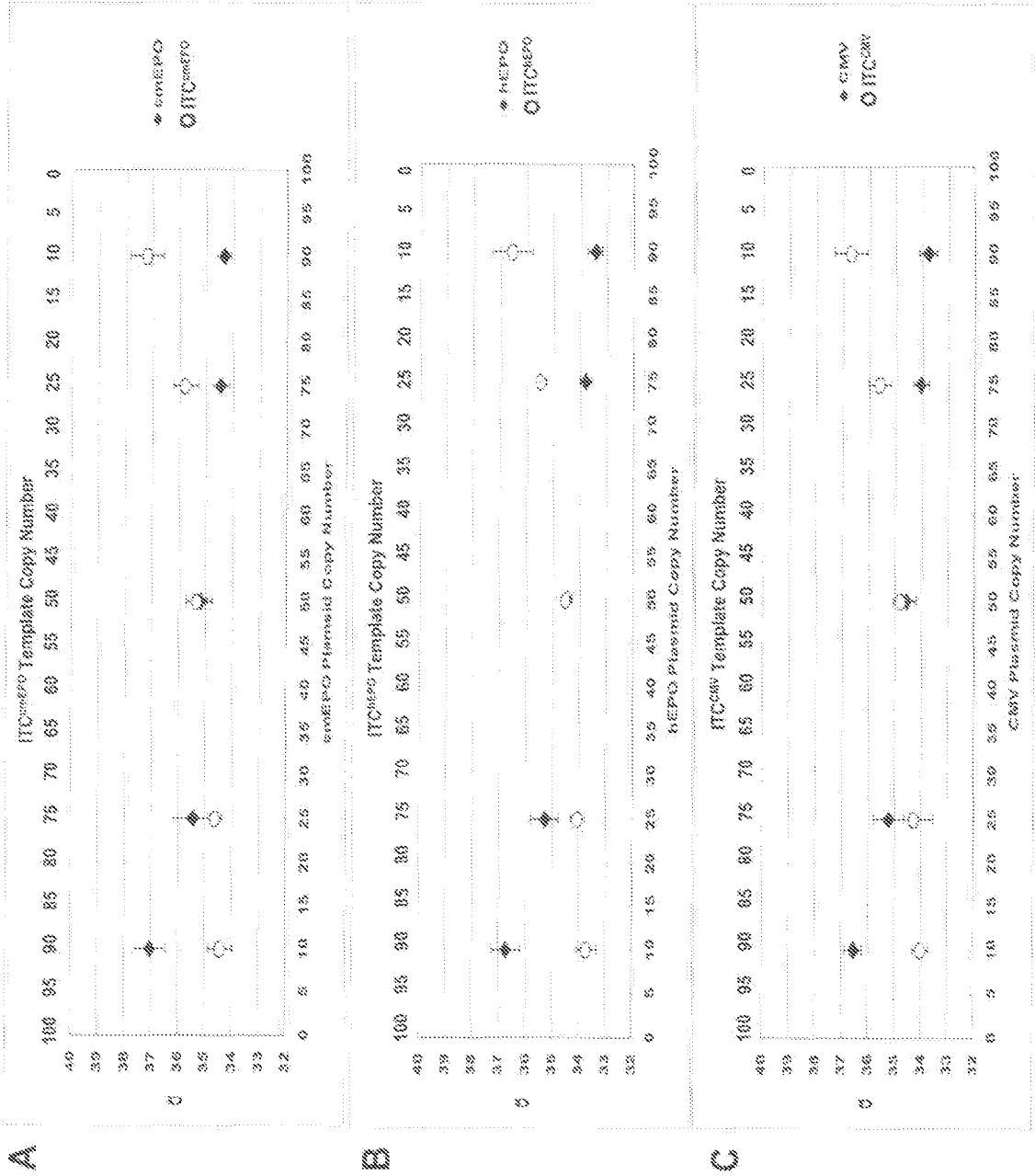
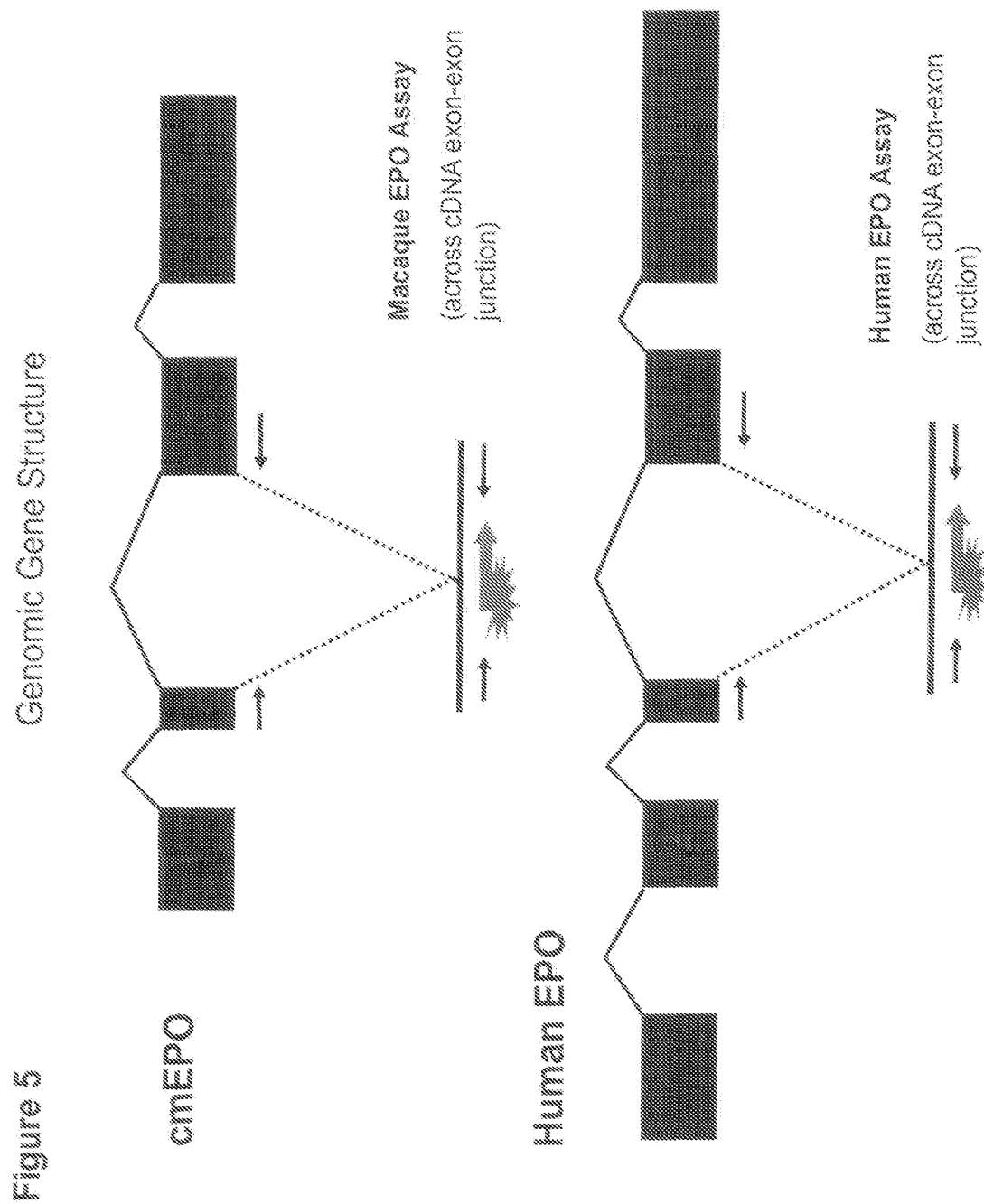


Figure 4







- (51) **International Patent Classification:**
G01N 33/53 (2006.01) *C12Q 1/68* (2006.01)
C12N 15/11 (2006.01) *G01N 33/52* (2006.01)
- (21) **International Application Number:**
PCT/US2012/030940
- (22) **International Filing Date:**
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- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
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- (63) **Related by continuation (CON) or continuation-in-part (CIP) to earlier application:**
US 61/468,361 (CON)
Filed on 28 March 2011 (28.03.2011)
- (71) **Applicant (for all designated States except US):**
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- (74) **Agents:** **ELRIFI, Ivor, R.** et al.; Mintz Levin Cohn Ferris
Glovsky And Popeo, P.C., One Financial Center, Boston,
MA 02111 (US).
- (81) **Designated States (unless otherwise indicated, for every
kind of national protection available):** AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD,
SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR,
TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

[Continued on next page]

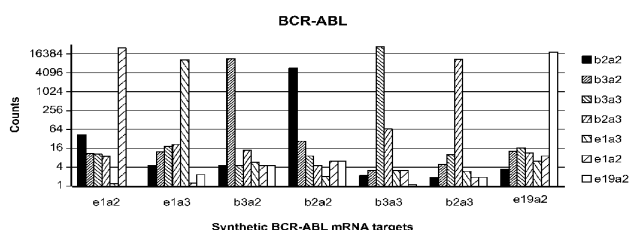
(54) **Title:** COMPOSITIONS AND METHODS FOR DIAGNOSING CANCER

FIG. 1A

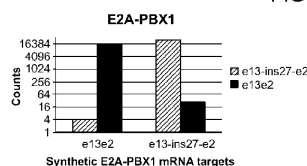


FIG. 1B

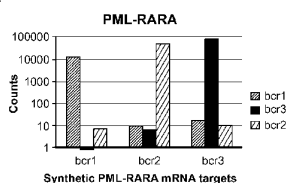


FIG. 1D

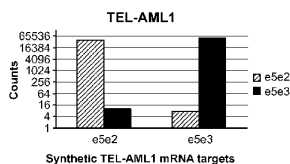


FIG. 1C

(57) **Abstract:** The application describes methods for diagnosing subjects with leukemia by detecting fusion genes associated with the onset of leukemia.



(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- *with international search report (Art. 21(3))*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

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20 December 2012

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/030940**A. CLASSIFICATION OF SUBJECT MATTER****G01N 33/53(2006.01)i, C12N 15/11(2006.01)i, C12Q 1/68(2006.01)i, G01N 33/52(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)

G01N 33/53; C07H 21/04; C07H 21/02; C40B 40/06; C12P 19/34; C12Q 1/68

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:leukemia, fusion gene, target-specific probe, plurality, label, nanoreporter.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2004/0235039 A1(GRAY, J.W. et al.) 25 Nov. 2004. See abstract, [0078], [0098], [0099], [0234]-[0240], [0333], [0356] and claim 127	1-25,51-53
Y	US 2010/0047924 A1(WEBSTER, P. J. et al.) 25 Feb. 2010. See the whole document, especially [0078] and figs. 1, 2	1-25,51-53
A	US 2003/0099987 A1 (WESTBROOK, C. A.) 29 May 2003 See [0036],[0038] and claims 1-3,5,14,21-23,29,30	1-25,51-53
A	US 2006/0134667 A1 (NARAHARA, M. et al.) 22 Jun 2006 See [0031],[0032],[0047],[0053] claims 1,9,10	1-25,51-53
A	US 6576421 B1 (WESTBROOK, C. A.) 10 Jun 2003 See column 7 lines 33-42 and claims 1,2,10,17,19,24,36	1-25,51-53
A	WO 2010/039275 A1 (OLIGONIX, INC.) 08 Apr 2010 See [00026] and claims 1,6,11,16	1-25,51-53

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

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

09 OCTOBER 2012 (09.10.2012)

Date of mailing of the international search report

10 OCTOBER 2012 (10.10.2012)

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, YUN-KYUNG

Telephone No. 82-42-481-5605



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2012/030940**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.: 26-50
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 26-50 pertain to a methods of diagnosis of the human body, thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.
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.:
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

Information on patent family members

International application No.

PCT/US2012/030940

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2004-0235039 A1	25. 11. 2004	CA 2131543 A1	16. 09. 1993
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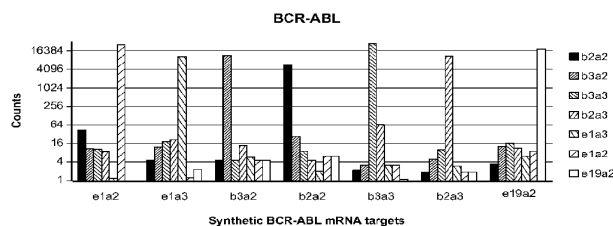


FIG. 1A

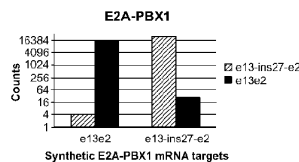


FIG. 1B

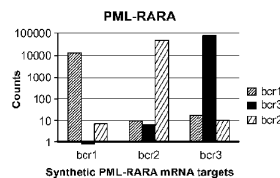


FIG. 1D

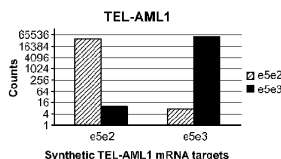


FIG. 1C

(57) Abstract: The application describes methods for dia-
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COMPOSITIONS AND METHODS FOR DIAGNOSING CANCER

CROSS-REFERENCE TO RELATED APPLICATIONS

[01] This application claims the benefit of U.S. Provisional Application No. 61/468,361, filed March 28, 2011, the contents of which are incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[02] This disclosure relates generally to the field of cancer biology, and specifically, to the fields of detection and identification of specific cancer cell phenotypes and correlation with relevant patient prognosis.

BACKGROUND OF THE INVENTION

[03] Recurrent chromosomal translocations characterize a substantial proportion of leukemias, resulting in the formation of unique fusion genes. The identification of particular fusion gene abnormalities is of considerable diagnostic and prognostic importance. There is a need in the art for reliable diagnostic compositions for the detection and diagnosis of leukemias and sub-types of leukemias based on gene fusion.

SUMMARY OF THE INVENTION

[04] Leukemia is often accompanied by the formation of fusion genes. These chromosomal abnormalities are caused by the reciprocal translocation of portions of genes at different chromosomal locations forming a new gene characterized by the fusion of a nucleic acid sequence from a first and a second gene. For example, the *BCR-ABL* fusion gene is caused by a reciprocal translocation of parts of chromosome 9 and 22. Specifically, the translocated regions are 9p34 and 22q11. This joins a portion of the *ABL1* gene on chromosome 9 to a part of the *BCR* ("breakpoint cluster region") of chromosome 22. The presence of this and other fusion genes is correlated with the onset and progression of various types of leukemia. This disclosure presents compositions and methods for the diagnosis and prognosis of leukemia based on the detection of fusion genes.

[05] The disclosure provides a composition for the diagnosis of leukemia. In one embodiment, this composition includes one or more target-specific nucleic acid probes. Each of

the one or more target-specific probes includes a target-specific region that specifically binds to one target nucleic acid analyte; and a region to which is attached a plurality of label monomers that create a unique label for each target-specific probe. The label has a detectable signal that distinguishes one target-specific probe which binds to a first target nucleic acid from another target-specific probe that binds to a different second target nucleic acid. One or more targets of the one or more target-specific probes include a fusion gene or mRNA transcribed from said fusion gene. The fusion gene is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia.

[06] The fusion genes can be caused by the fusion of two or more genes at the onset of leukemia. The fusion gene can be *BCR-ABL*, *AML-ETO*, *CBFB-MYH11*, *E2A-PBX1*, *MLL-AF4*, *PML-RARA*, or *TEL-AML1*. The fusions can be between varying exons of the upstream and downstream genes. The leukemia can be chronic myelogenous leukemia, acute myeloid leukemia or acute lymphoblastic leukemia.

[07] In certain embodiments, at least one of the one or more target-specific probes has a target that is the sequence of the junction where the two genes are fused. These target-specific probes can also include a target-specific region with one or more mismatches to the target of the target-specific region.

[08] In other specific embodiments, the label monomers can be a fluorochrome moiety, a fluorescent moiety, a dye moiety or a chemiluminescent moiety. The unique label for each target-specific probe can be composed of 4 or 5 label monomers.

[09] The disclosure also provides a composition for the diagnosis of leukemia and different sub-types of leukemia. In one embodiment, the composition comprises a probe pair including a first probe and a second probe. The first probe is made up, at least in part, of a complex including a first molecule. The first molecule includes a first label attachment region to which are attached one or more label monomers that emit light constituting a first signal. The first molecule also includes a second label attachment region, which is non-overlapping with the first label attachment region, to which are attached one or more label monomers that emit light constituting a second signal. The first molecule also includes a first target-specific sequence attached to the first molecule. The second probe is made up, at least in part, of a second molecule including a second target-specific sequence and an affinity tag. The first target-specific sequence and the second target-specific sequence bind to different regions of the same target

molecule. The target molecule is a fusion gene or mRNA transcribed from said fusion gene. The fusion gene is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia. Also, when said probe pair is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitute at least part of a code that identifies the target molecule.

[10] In certain embodiments, the fusion gene results from the fusion of at least a portion of a first gene and at least a portion of a second gene. In this situation, the first target-specific sequence can hybridize to a portion of the first gene and the second target-specific sequence can hybridize to a portion of the second gene. The first target-specific sequence can hybridize to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene.

[11] In other embodiments, the first target-specific sequence can also hybridize to a portion of the first gene and a portion of the second gene. In this situation, the first target-specific sequence can hybridize to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene. At least one of the target-specific sequences can include a region with one or more mismatches to the target of the target-specific sequence.

[12] The second target-specific sequence can also hybridize to a portion of the first gene and a portion of the second gene. In this situation, the first target-specific sequence can hybridize to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene. At least one of the target-specific sequences can include a region with one or more mismatches to the target of the target-specific sequence.

[13] The leukemia can be chronic myelogenous leukemia, acute myeloid leukemia or acute lymphoblastic leukemia. The fusion gene can be *BCR-ABL*, *AML-ETO*, *CBFB-MYH11*, *E2A-PBX1*, *MLL-AF4*, *PML-RARA*, or *TEL-AML1*.

[14] In certain embodiments, each of said label monomers can be a fluorochrome moiety, a fluorescent moiety, a dye moiety or a chemiluminescent moiety. The unique label for each target-specific probe can be composed of 4 or 5 label monomers.

[15] The disclosure also provides a method for diagnosing leukemia, a specific subtype of leukemia or determining a genetic aberration underlying leukemia in a subject. In one embodiment, the method includes providing a sample from the subject and contacting the sample with one or more target-specific probes. Each of the one or more target-specific probes includes

a target-specific region that binds to one target nucleic acid analyte; and a region to which is attached a plurality of label monomers that create a unique label for each target-specific probe. The label has a detectable signal that distinguishes one target-specific probe which binds to a first target nucleic acid from another target-specific probe that binds to a different second target nucleic acid. One or more targets of the one or more target-specific probes include a fusion gene or mRNA transcribed from said fusion gene. The fusion gene is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia. When the fusion gene is detected the subject is diagnosed with leukemia, a specific subtype of leukemia or the genetic aberration underlying leukemia in a subject is determined.

[16] The fusion gene can be *BCR-ABL*, *AML-ETO*, *CBFB-MYH11*, *E2A-PBX1*, *MLL-AF4*, *PML-RARA*, or *TEL-AML1*. The leukemia can be chronic myelogenous leukemia, acute myeloid leukemia or acute lymphoblastic leukemia.

[17] In certain embodiments, at least one of the one or more target-specific probes can have a target that is the sequence of the junction of the two genes that are fused. The one or more target-specific probes can include a target-specific region with one or more mismatches to the target of the target-specific region.

[18] In other embodiments, each of the label monomers can be a fluorochrome moiety, a fluorescent moiety, a dye moiety or a chemiluminescent moiety. The unique label for each target-specific probe can be composed of 4 or 5 label monomers.

[19] The disclosure also provides a method for diagnosing leukemia, a specific subtype of leukemia or determining a genetic aberration underlying leukemia in a subject. In one embodiment, the method includes providing a sample from the subject and contacting the sample with a probe pair. The probe pair includes a first probe and a second probe. The first probe is made up, at least in part, of a complex including a first molecule with a first label attachment region to which are attached one or more label monomers that emit light constituting a first signal. The first probe also includes a second label attachment region, which is non-overlapping with the first label attachment region, to which are attached one or more label monomers that emit light constituting a second signal. The first probe also includes a first target-specific sequence attached to the first molecule. The second probe is made up, at least in part, of a second molecule, comprising a second target-specific sequence and an affinity tag. The first target-specific sequence and the second target-specific sequence bind to different regions of the

same target molecule. The target molecule is a fusion gene or mRNA transcribed from said fusion gene. The fusion gene is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia. When said probe pair is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitute at least part of a code that identifies the target molecule. Also, when the fusion gene is detected the subject is diagnosed with leukemia, a specific subtype of leukemia or the genetic aberration underlying leukemia is determined.

[20] In certain embodiments, the fusion gene can include the fusion of at least a portion of a first gene and at least a portion of a second gene. The first target-specific sequence can hybridize to a portion of the first gene and the second target-specific sequence hybridizes to a portion of the second gene. The first target-specific sequence can also hybridize to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene.

[21] In other embodiments, the first target-specific sequence can hybridize to a portion of the first gene and a portion of the second gene. The first target-specific sequence can also hybridize to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene. At least one of the target-specific sequences can include a region with one or more mismatches to the target of the target-specific sequence.

[22] In other embodiments, the second target-specific sequence can hybridize to a portion of the first gene and a portion of the second gene. The first target-specific sequence can also hybridize to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene. At least one of the target-specific sequences can include a region with one or more mismatches to the target of the target-specific sequence.

[23] The leukemia can be chronic myelogenous leukemia, acute myeloid leukemia or acute lymphoblastic leukemia. The fusion gene can be *BCR-ABL*, *AML-ETO*, *CBFB-MYH11*, *E2A-PBX1*, *MLL-AF4*, *PML-RARA*, or *TEL-AML1*.

[24] Each of said label monomers can be a fluorochrome moiety, a fluorescent moiety, a dye moiety or a chemiluminescent moiety. The unique label for each target-specific probe can be composed of 4 or 5 label monomers.

[25] The disclosure also provides a kit. In one embodiment, the kit includes one or more reporter probes and one or more capture probes. The reporter probe and the capture probe each

bind specifically to a fusion gene. The fusion gene is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia.

[26] The fusion gene can be *BCR-ABL*, *AML-ETO*, *CBFB-MYH11*, *E2A-PBX1*, *MLL-AF4*, *PML-RARA*, or *TEL-AML1*. The kit can include one or more reporter probes and one or more capture probes from Table 2.

BRIEF DESCRIPTION OF THE DRAWINGS

[27] Figures 1A is a bar graph showing counts for probes for the *BCR-ABL* gene fusion when exposed to gene fragments of the *BCR-ABL* gene fusion and non-specific targets.

[28] Figure 1B is a bar graph showing counts for probes for the *E2A-PBX1* gene fusion when exposed to gene fragments of the *E2A-PBX1* gene fusion and non-specific targets.

[29] Figure 1C is a bar graph showing counts for probes for the *TEL-AML1* gene fusion when exposed to gene fragments of the *TEL-AML1* gene fusion and non-specific targets.

[30] Figure 1D is a bar graph showing counts for probes for the *PML-RARA* gene fusion when exposed to gene fragments of the *PML-RARA* gene fusion and non-specific targets.

[31] Figure 2A is a bar graph showing the relative expression of various gene fusions in the SUP B13 cell line.

[32] Figure 2B is a bar graph showing the relative expression of various gene fusions in the K562 cell line.

[33] Figure 2C is a bar graph showing the relative expression of various gene fusions in the NB4 cell line.

[34] Figure 2D is a bar graph showing the relative expression of various gene fusions in the Kasumi cell line.

[35] Figure 2E is a bar graph showing the relative expression of various gene fusions in the KCL-22 cell line.

[36] Figure 2F is a bar graph showing the relative expression of various gene fusions in the MV4-11 cell line.

DETAILED DESCRIPTION OF THE INVENTION

[37] This disclosure describes compositions and methods for the diagnosis of leukemia, diagnosis of the subtype of leukemia present in a subject and determination of the genetic

aberration underlying leukemia in a subject. The onset and progression of leukemia is associated with the formation of several fusion genes. These fusion genes include *BCR-ABL*, *AML-ETO*, *CBFB-MYH11*, *E2A-PBX1*, *MLL-AF4*, *PML-RARA*, or *TEL-AML1*. The compositions disclosed herein are nucleic acid probes used to detect the presence of these fusion genes.

[38] The detection of these fusion genes can be used to detect the presence of leukemia and also what type of leukemia is present. For example, a *BCR-ABL* fusion gene is associated with chronic myelogenous leukemia. *AML-ETO*, *CBFB-MYH11*, *MLL-AF4*, and *PML-RARA* fusion genes are associated with acute myeloid leukemia. *E2A-PBX1* and *TEL-AML1* fusion genes are associated with acute lymphoblastic leukemia. In addition, specific isoforms of a given fusion may indicate a specific sub-type of disease.

[39] The presence of certain gene fusions in certain types of leukemia is associated with differing prognoses in the outcome of the disease for a subject. Thus, the detection of various gene fusions can be used to provide a prognosis for a subject with leukemia.

[40] Preferably, the nucleic acid probes used according to the methods of the disclosure are nanoreporters. A fully assembled and labeled nanoreporter comprises two main portions, a target-specific sequence that is capable of binding to a target molecule, and a labeled region which emits a “code” of signals (the “nanoreporter code”) associated with the target-specific sequence.

[41] Upon binding of the nanoreporter to the target molecule, the nanoreporter code identifies the target molecule to which the nanoreporter is bound.

Nanoreporters

[42] Many nanoreporters, referred to as singular nanoreporters, are composed of one molecular entity. However, to increase the specificity of a nanoreporter and/or to improve the kinetics of its binding to a target molecule, a preferred nanoreporter is a dual nanoreporter composed of two molecular entities, each containing a different target-specific sequence that binds to a different region of the same target molecule. In a dual nanoreporter, at least one of the two nanoreporter probes is labeled. This labeled nanoreporter probe is referred to herein as a “reporter probe”. The other nanoreporter probe is not necessarily labeled. Such unlabeled components of dual nanoreporters are referred to herein as “capture probes” and often have affinity tags attached, such as biotin, which are useful to immobilize and/or stretch the complex containing the dual nanoreporter and the target molecule to allow visualization and/or imaging of

the complex. When both probes are labeled or both have affinity tags, the probe with more label monomer attachment regions is referred to as the reporter probe and the other probe in the pair is referred to as a capture probe.

[43] For both single and dual nanoreporters, a fully assembled and labeled nanoreporter probe comprises two main portions, a target-specific sequence that is capable of binding to a target molecule, and a labeled portion which provides a "code" of signals associated with the target-specific sequence. Upon binding of the nanoreporter probe to the target molecule, the code identifies the target molecule to which the nanoreporter is bound.

[44] Nanoreporters are modular structures. In some embodiments, the nanoreporter comprises a plurality of different detectable molecules. In some embodiments, a labeled nanoreporter, is a molecular entity containing certain basic elements: (i) a plurality of unique label attachment regions attached in a particular, unique linear combination, and (ii) complementary polynucleotide sequences attached to the label attachment regions of the backbone. In some embodiments, the labeled nanoreporter comprises 2, 3, 4, 5, 6, 7, 8, 9, 10 or more unique label attachment regions attached in a particular, unique linear combination, and complementary polynucleotide sequences attached to the label attachment regions of the backbone. In some embodiments, the labeled nanoreporter comprises 6 or more unique label attachment regions attached in a particular, unique linear combination, and complementary polynucleotide sequences attached to the label attachment regions of the backbone. A nanoreporter probe further comprises a target-specific sequence, also attached to the backbone.

[45] The term label attachment region includes a region of defined polynucleotide sequence within a given backbone that may serve as an individual attachment point for a detectable molecule. In some embodiments, the label attachment regions comprise designed sequences.

[46] In some embodiments, the label nanoreporter also comprises a backbone containing a constant region. The term constant region includes tandemly-repeated sequences of about 10 to about 25 nucleotides that are covalently attached to a nanoreporter. The constant region can be attached at either the 5' region or the 3' region of a nanoreporter, and may be utilized for capture and immobilization of a nanoreporter for imaging or detection, such as by attaching to a solid substrate a sequence that is complementary to the constant region. In certain aspects, the constant region contains 2, 3, 4, 5, 6, 7, 8, 9, 10, or more tandemly-repeated sequences, wherein the repeat sequences each comprise about 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 nucleotides, including about 12-18, 13-17, or about 14-16 nucleotides.

[47] The nanoreporters described herein can comprise synthetic, designed sequences. In some embodiments, the sequences contain a fairly regularly-spaced pattern of a nucleotide (e.g. adenine) residue in the backbone. In some embodiments, a nucleotide is spaced at least an average of 8, 9, 10, 12, 15, 16, 20, 30, or 50 bases apart. In some embodiments, a nucleotide is spaced at least an average of 8 to 16 bases apart. In some embodiments, a nucleotide is spaced at least an average of 8 bases apart. This allows for a regularly spaced complementary nucleotide in the complementary polynucleotide sequence having attached thereto a detectable molecule. For example, in some embodiments, when the nanoreporter sequences contain a fairly regularly-spaced pattern of adenine residues in the backbone, whose complement is a regularly-spaced pattern of uridine (U) residues in complementary RNA segments, the in vitro transcription of the segments can be done using an aminoallyl-modified uridine base, which allows the covalent amine coupling of dye molecules at regular intervals along the segment. In some embodiments, the sequences contain about the same number or percentage of a nucleotide (e.g. adenine) that is spaced at least an average of 8, 9, 10, 12, 15, 16, 20, 30, or 50 bases apart in the sequences. This allows for similar number or percentages in the complementary polynucleotide sequence having attached thereto a detectable molecule. Thus, in some embodiments, the sequences contain a nucleotide that is not regularly-spaced but that is spaced at least an average of 8, 9, 10, 12, 15, 16, 20, 30, or 50 bases apart. In some embodiments, 20%, 30%, 50%, 60%, 70%, 80%, 90% or 100% of the complementary nucleotide is coupled to a detectable molecule. For instance, in some embodiments, when the nanoreporter sequences contain a similar percentage of adenine residues in the backbone and the in vitro transcription of the complementary segments is done using an aminoallyl-modified uridine base, 20%, 30%, 50%, 60%, 70%, 80%, 90% or 100% of the aminoallyl-modified uridine base can be coupled to a detectable molecule. Alternatively, the ratio of aminoallyl-modified uridine bases and uridine bases can be changed during the in vitro transcription process to achieve the desired number of sites which can be attached to a detectable molecule. For example, in vitro transcription process can take place in the presence of a mixture with a ratio of 1/1 of uridine to aminoallyl-modified uridine bases, when some or all the aminoallyl-modified uridine bases can be coupled to a detectable molecule.

[48] In some embodiments, the nanoreporters described herein have a fairly consistent melting

temperature (T_m). Without intending to be limited to any theory, the T_m of the nanoreporters described herein provides for strong bonds between the nanoreporter backbone and the complementary polynucleotide sequence having attached thereto a detectable molecule, therefore, preventing dissociation during synthesis and hybridization procedures. In addition, the consistent T_m among a population of nanoreporters allows for the synthesis and hybridization procedures to be tightly optimized, as the optimal conditions are the same for all spots and positions. In some embodiments, the sequences of the nanoreporters have a 50% guanine/cytosine (G/C), with no more than three G's in a row. Thus, in some embodiments, the disclosure provides a population of nanoreporters in which the T_m among the nanoreporters in the population is fairly consistent. In some embodiments, the disclosure provides a population of nanoreporters in which the T_m of the complementary polynucleotide sequences when hybridized to its label attachment regions is about 80° C, 85° C, 90° C, 100° C or higher. In some embodiments, the disclosure provides a population of nanoreporters in which the T_m of the complementary polynucleotide sequences when hybridized to its label attachment regions is about 80° C or higher.

[49] In some embodiments, the nanoreporters described herein have minimal or no secondary structures, such as any stable intra-molecular base-pairing interaction (e.g. hairpins). Without intending to be limited to any theory, the minimal secondary structure in the nanoreporters provides for better hybridization between the nanoreporter backbone and the polynucleotide sequence having attached thereto a detectable molecule. In addition, the minimal secondary structure in the nanoreporters provides for better detection of the detectable molecules in the nanoreporters. In some embodiments, the nanoreporters described herein have no significant intra-molecular pairing under annealing conditions of 75° C, 1 X SSPE. Secondary structures can be predicted by programs known in the art such as MFOLD. In some embodiments, the nanoreporters described herein contain less than 1% of inverted repeats in each strand, wherein the inverted repeats are 9 bases or greater. In some embodiments, the nanoreporters described herein contain no inverted repeats in each strand. In some embodiments, the nanoreporters do not contain any inverted repeat of 9 nucleotides or greater across a sequence that is 1100 base pairs in length. In some embodiments, the nanoreporters do not contain any inverted repeat of 7 nucleotides or greater across any 100 base pair region. In some embodiments, the nanoreporters described herein contain less than 1% of inverted repeats in each strand, wherein the inverted

repeats are 9 nucleotides or greater across a sequence that 1100 base pairs in length. In some embodiments, the nanoreporters described herein contain less than 1% of inverted repeats in each strand, wherein the inverted repeats are 7 nucleotides or greater across any 100 base pair region. In some embodiments, the nanoreporters described herein contain a skewed strand specific content such that one strand is CT-rich and the other is GA-rich.

[50] The disclosure also provides unique nanoreporters. In some embodiments, the nanoreporters described herein contain less than 1% of direct repeats. In some embodiments, the nanoreporters described herein contain no direct repeats. In some embodiments, the nanoreporters do not contain any direct repeat of 9 nucleotides or greater across a sequence that 1100 base pairs in length. In some embodiments, the labeled nanoreporters do not contain any direct repeat of 7 nucleotides or greater across any 100 base pair region. In some embodiments, the nanoreporters described herein contain less than 1% of direct repeats in each strand, wherein the direct repeats are 9 nucleotides or greater across a sequence that 1100 base pairs in length. In some embodiments, the nanoreporters described herein contain less than 1% of direct repeats in each strand, wherein the direct repeats are 7 nucleotides or greater across any 100 base pair region. In some embodiments, the nanoreporters described herein contain less than 85, 80, 70, 60, 50, 40, 30, 20, 10, or 5% homology to any other sequence used in the backbones or to any sequence described in the REFSEQ public database. In some embodiments, the nanoreporters described herein contain less than 85% homology to any other sequence used in the backbones or to any sequence described in the REFSEQ public database. In some embodiments, the nanoreporters described herein contain less than 20, 16, 15, 10, 9, 7, 5, 3, 2 contiguous bases of homology to any other sequence used in the backbones or to any sequence described in the REFSEQ public database. In some embodiments, the nanoreporters described herein have no more than 15 contiguous bases of homology and no more than 85% identity across the entire length of the nanoreporter to any other sequence used in the backbones or to any sequence described in the REFSEQ public database.

[51] In some embodiments, the sequence characteristics of the nanoreporter probes described herein provide sensitive detection of a target molecule. For instance, the binding of the nanoreporter probes to target molecules which results in the identification of the target molecules can be performed by individually detecting the presence of the nanoreporter. This can be performed by individually counting the presence of one or more of the nanoreporter molecules in

a sample.

[52] The complementary polynucleotide sequences attached to a nanoreporter backbone serve to attach detectable molecules, or label monomers, to the nanoreporter backbone. The complementary polynucleotide sequences may be directly labeled, for example, by covalent incorporation of one or more detectable molecules into the complementary polynucleotide sequence. Alternatively, the complementary polynucleotide sequences may be indirectly labeled, such as by incorporation of biotin or other molecule capable of a specific ligand interaction into the complementary polynucleotide sequence. In such instances, the ligand (e.g., streptavidin in the case of biotin incorporation into the complementary polynucleotide sequence) may be covalently attached to the detectable molecule. Where the detectable molecules attached to a label attachment region are not directly incorporated into the complementary polynucleotide sequence, this sequence serves as a bridge between the detectable molecule and the label attachment region, and may be referred to as a bridging molecule, e.g., a bridging nucleic acid.

[53] The nucleic-acid based nanoreporter and nanoreporter-target complexes described herein comprise nucleic acids, which may be affinity-purified or immobilized using a nucleic acid, such as an oligonucleotide, that is complementary to the constant region or the nanoreporter or target nucleic acid. As noted above, in some embodiments the nanoreporters comprise at least one constant region, which may serve as an affinity tag for purification and/or for immobilization (for example to a solid surface). The constant region typically comprises two or more tandemly-repeated regions of repeat nucleotides, such as a series of 15-base repeats. In such exemplary embodiments, the nanoreporter, whether complexed to a target molecule or otherwise, can be purified or immobilized by an affinity reagent coated with a 15-base oligonucleotide which is the reverse complement of the repeat unit.

[54] Nanoreporters, or nanoreporter-target molecule complexes, can be purified in two or more affinity selection steps. For example, in a dual nanoreporter, one probe can comprise a first affinity tag and the other probe can comprise a second (different) affinity tag. The probes are mixed with target molecules, and complexes comprising the two probes of the dual nanoreporter are separated from unbound materials (e.g., the target or the individual probes of the nanoreporter) by affinity purification against one or both individual affinity tags. In the first step, the mixture can be bound to an affinity reagent for the first affinity tag, so that only probes comprising the first affinity tag and the desired complexes are purified. The bound materials are

released from the first affinity reagent and optionally bound to an affinity reagent for the second affinity tag, allowing the separation of complexes from probes comprising the first affinity tag. At this point only full complexes would be bound. The complexes are finally released from the affinity reagent for the second affinity tag and then preferably stretched and imaged. The affinity reagent can be any solid surface coated with a binding partner for the affinity tag, such as a column, bead (e.g., latex or magnetic bead) or slide coated with the binding partner.

Immobilizing and stretching nanoreporters using affinity reagents is fully described in U.S. Publication No. 2010/0161026, which is incorporated by reference herein in its entirety.

[55] The sequence of signals provided by the label monomers associated with the various label attachment regions of the backbone of a given nanoreporter allows for the unique identification of the nanoreporter. For example, when using fluorescent labels, a nanoreporter having a unique identity or unique spectral signature is associated with a target-specific sequence that recognizes a specific target molecule or a portion thereof. When a nanoreporter is exposed to a mixture containing the target molecule under conditions that permit binding of the target-specific sequence(s) of the nanoreporter to the target molecule, the target-specific sequence(s) preferentially bind(s) to the target molecule. Detection of the nanoreporter signal, such as the spectral code of a fluorescently labeled nanoreporter, associated with the nanoreporter allows detection of the presence of the target molecule in the mixture (qualitative analysis). Counting all the label monomers associated with a given spectral code or signature allows the counting of all the molecules in the mixture associated with the target-specific sequence coupled to the nanoreporter (quantitative analysis). Nanoreporters are thus useful for the diagnosis or prognosis of different biological states (e.g., disease vs. healthy) by quantitative analysis of known biological markers. Moreover, the exquisite sensitivity of single molecule detection and quantification provided by the nanoreporters described herein allows for the identification of new diagnostic and prognostic markers, including those whose fluctuations among the different biological states is too slight to detect a correlation with a particular biological state using traditional molecular methods. The sensitivity of nanoreporter-based molecular detection permits detailed pharmacokinetic analysis of therapeutic and diagnostic agents in small biological samples.

[56] Many nanoreporters, referred to as singular nanoreporters, are composed of one molecular entity. However, to increase the specificity of a nanoreporter and/or to improve the

kinetics of its binding to a target molecule, a nanoreporter can be a dual nanoreporter composed of two molecular entities, each containing a different target-specific sequence that binds to a different region of the same target molecule. In a dual nanoreporter, at least one of the two molecular entities is labeled. The other molecular entity need not necessarily be labeled. Such unlabeled components of dual nanoreporters may be used as capture probes and optionally have affinity tags attached, such as biotin, which are useful to immobilize and/or stretch the complex containing the dual nanoreporter and the target molecule to allow visualization and/or imaging of the complex. For instance, in some embodiments, a dual nanoreporter with a 6-position nanoreporter code uses one 6-position coded nanoreporter (also referred to herein as a reporter probe) and a capture probe. In some embodiments, a dual nanoreporter with a 6-position nanoreporter code can be used, using one capture probe with an affinity tag and one 6-position nanoreporter component. In some embodiments an affinity tag is optionally included and can be used to purify the nanoreporter or to immobilize the nanoreporter (or nanoreporter-target molecule complex) for the purpose of imaging.

[57] In some embodiments, the nucleotide sequences of the individual label attachment regions within each nanoreporter are different from the nucleotide sequences of the other label attachment regions within that nanoreporter, preventing rearrangements, such as recombination, sharing or swapping of the label polynucleotide sequences. The number of label attachment regions to be formed on a backbone is based on the length and nature of the backbone, the means of labeling the nanoreporter, as well as the type of label monomers providing a signal to be attached to the label attachment regions of the backbone. In some embodiments, the complementary nucleotide sequence of each label attachment region is assigned a specific detectable molecule.

[58] The disclosure also provides labeled nanoreporters wherein one or more label attachment regions are attached to a corresponding detectable molecule, each detectable molecule providing a signal. For example, in some embodiments, a labeled nanoreporter according to the disclosure is obtained when at least three detectable molecules are attached to three corresponding label attachment regions of the backbone such that these labeled label attachment regions, or spots, are distinguishable based on their unique linear arrangement. A "spot," in the context of nanoreporter detection, is the aggregate signal detected from the label monomers attached to a single label attachment site on a nanoreporter, and which, depending on the size of the label attachment

region and the nature (e.g., primary emission wavelength) of the label monomer, may appear as a single point source of light when visualized under a microscope. Spots from a nanoreporter may be overlapping or non-overlapping. The nanoreporter code that identifies that target molecule can comprise any permutation of the length of a spot, its position relative to other spots, and/or the nature (e.g., primary emission wavelength(s)) of its signal. Generally, for each probe or probe pair described herein, adjacent label attachment regions are non-overlapping, and/or the spots from adjacent label attachment regions are spatially and/or spectrally distinguishable, at least under the detection conditions (e.g., when the nanoreporter is immobilized, stretched and observed under a microscope, as described in U.S. Publication No. 2010/0112710, incorporated herein by reference).

[59] Occasionally, reference is made to a spot size as a certain number of bases or nucleotides. As would be readily understood by one of skill in the art, this refers to the number of bases or nucleotides in the corresponding label attachment region.

[60] The order and nature (e.g., primary emission wavelength(s), optionally also length) of spots from a nanoreporter serve as a nanoreporter code that identifies the target molecule capable of being bound by the nanoreporter through the nanoreporter's target specific sequence(s). When the nanoreporter is bound to a target molecule, the nanoreporter code also identifies the target molecule. Optionally, the length of a spot can be a component of the nanoreporter code.

[61] Detectable molecules providing a signal associated with different label attachment regions of the backbone can provide signals that are indistinguishable under the detection conditions ("like" signals), or can provide signals that are distinguishable, at least under the detection conditions (e.g., when the nanoreporter is immobilized, stretched and observed under a microscope).

[62] The disclosure also provides a nanoreporter wherein two or more detectable molecules are attached to a label attachment region. The signal provided by the detectable molecules associated with said label attachment region produces an aggregate signal that is detected. The aggregate signal produced may be made up of like signals or made up of at least two distinguishable signals (e.g., spectrally distinguishable signals).

[63] In one embodiment, a nanoreporter includes at least three detectable molecules providing like signals attached to three corresponding label attachment regions of the backbone and said three detectable molecules are spatially distinguishable. In another embodiment, a nanoreporter

includes at least three detectable molecules providing three distinguishable signals attached to three neighboring label attachment regions, for example three adjacent label attachment regions, whereby said at least three label monomers are spectrally distinguishable.

[64] In other embodiments, a nanoreporter includes spots providing like or unlike signals separated by a spacer region, whereby interposing the spacer region allows the generation of dark spots, which expand the possible combination of uniquely detectable signals. The term "dark spot" refers to a lack of signal from a label attachment site on a nanoreporter. Dark spots can be incorporated into the nanoreporter code to add more coding permutations and generate greater nanoreporter diversity in a nanoreporter population. In one embodiment, the spacer regions have a length determined by the resolution of an instrument employed in detecting the nanoreporter.

[65] In other embodiments, a nanoreporter includes one or more "double spots." Each double spot contains two or more (e.g., three, four or five) adjacent spots that provide like signals without being separated by a spacer region. Double spots can be identified by their sizes.

[66] A detectable molecule providing a signal described herein may be attached covalently or non-covalently (e.g., via hybridization) to a complementary polynucleotide sequence that is attached to the label attachment region. The label monomers may also be attached indirectly to the complementary polynucleotide sequence, such as by being covalently attached to a ligand molecule (e.g., streptavidin) that is attached through its interaction with a molecule incorporated into the complementary polynucleotide sequence (e.g., biotin incorporated into the complementary polynucleotide sequence), which is in turn attached via hybridization to the backbone.

[67] A nanoreporter can also be associated with a uniquely detectable signal, such as a spectral code, determined by the sequence of signals provided by the label monomers attached (e.g., indirectly) to label attachment regions on the backbone of the nanoreporter, whereby detection of the signal allows identification of the nanoreporter.

[68] In other embodiments, a nanoreporter also includes an affinity tag attached to the reporter probe backbone, such that attachment of the affinity tag to a support allows backbone stretching and resolution of signals provided by label monomers corresponding to different label attachment regions on the backbone. Nanoreporter stretching may involve any stretching means known in the art including but not limited to, means involving physical, hydrodynamic or

electrical means. The affinity tag may comprise a constant region.

[69] In other embodiments, a nanoreporter also includes a target-specific sequence coupled to the backbone. The target-specific sequence is selected to allow the nanoreporter to recognize, bind or attach to a target molecule. The nanoreporters described herein are suitable for identification of target molecules of all types. For example, appropriate target-specific sequences can be coupled to the backbone of the nanoreporter to allow detection of a target molecule. Preferably the target molecule is DNA (including cDNA), RNA (including mRNA and cRNA), a peptide, a polypeptide, or a protein.

[70] One embodiment of the disclosure provides increased flexibility in target molecule detection with label monomers described herein. In this embodiment, a dual nanoreporter comprising two different molecular entities, each with a separate target-specific region, at least one of which is labeled, bind to the same target molecule. Thus, the target-specific sequences of the two components of the dual nanoreporter bind to different portions of a selected target molecule, whereby detection of the spectral code associated with the dual nanoreporter provides detection of the selected target molecule in a biomolecular sample contacted with said dual nanoreporter.

[71] The disclosure also provides a method of detecting the presence of a specific target molecule in a biomolecular sample comprising: (i) contacting said sample with a nanoreporter as described herein (e.g., a singular or dual nanoreporter) under conditions that allow binding of the target-specific sequences in the dual nanoreporter to the target molecule and (ii) detecting the spectral code associated with the dual nanoreporter. Depending on the nanoreporter architecture, the dual nanoreporter may be labeled before or after binding to the target molecule.

[72] The uniqueness of each nanoreporter probe in a population of probe allows for the multiplexed analysis of a plurality of target molecules. For example, in some embodiments, each nanoreporter probe contains six label attachment regions, where each label attachment region of each backbone is different from the other label attachment regions in that same backbone. If the label attachment regions are going to be labeled with one of four colors and there are 24 possible unique sequences for the label attachment regions and each label attachment region is assigned a specific color, each label attachment region in each backbone will consist of one of four sequences. There will be 4096 possible nanoreporters in this example. The number of possible nanoreporters can be increased, for example, by increasing the number of colors, increasing the

number of unique sequences for the label attachment regions and/or increasing the number of label attachment regions per backbone. Likewise the number of possible nanoreporters can be decreased by decreasing the number of colors, decreasing the number of unique sequences for the label attachment regions and/or decreasing the number of label attachment regions per backbone.

[73] In certain embodiments, the methods of detection are performed in multiplex assays, whereby a plurality of target molecules are detected in the same assay (a single reaction mixture). In a preferred embodiment, the assay is a hybridization assay in which the plurality of target molecules are detected simultaneously. In certain embodiments, the plurality of target molecules detected in the same assay is, at least 2, at least 5 different target molecules, at least 10 different target molecules, at least 20 different target molecules, at least 50 different target molecules, at least 75 different target molecules, at least 100 different target molecules, at least 200 different target molecules, at least 500 different target molecules, or at least 750 different target molecules, or at least 1000 different target molecules. In other embodiments, the plurality of target molecules detected in the same assay is up to 50 different target molecules, up to 100 different target molecules, up to 150 different target molecules, up to 200 different target molecules, up to 300 different target molecules, up to 500 different target molecules, up to 750 different target molecules, up to 1000 different target molecules, up to 2000 different target molecules, or up to 5000 different target molecules. In yet other embodiments, the plurality of target molecules detected is any range in between the foregoing numbers of different target molecules, such as, but not limited to, from 20 to 50 different target molecules, from 50 to 200 different target molecules, from 100 to 1000 different target molecules, from 500 to 5000 different target molecules, and so on and so forth.

[74] In certain embodiments, nanoreporters for detecting the presence of fusion genes contain both a reporter probe and a capture probe that hybridize to the fusion gene at different positions. In one embodiment, the target-specific regions of the capture and reporter probe hybridize to sequences on either side of the border of the fusion between the two fused genes. Optionally, the target-specific regions hybridize to regions adjacent to each other on either side of the border of fusion. The sequences to which the target-specific regions of the reporter and capture probes hybridize can also be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more base pairs apart.

[75] In another embodiment, the reporter probe's target-specific region hybridizes to a sequence that spans the fusion junction while the target-specific region of the capture probe hybridizes to a sequence upstream or downstream of the reporter probe's target. In another embodiment, the capture probe's target-specific region hybridizes to a sequence that spans the fusion junction while the target-specific region of the reporter probe hybridizes to a sequence upstream or downstream of the reporter probe's target. Optionally, the target sequences of the reporter and capture probes are adjacent. The sequences to which the target-specific regions of the reporter and capture probes hybridize can also be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more base pairs apart. In certain embodiments, the target-specific region of a probe has 1, 2, 3, 4, 5 or more mismatches introduced into the target-specific sequence. The sequences which come together to form the junction of a fusion gene can be GC rich, resulting in a high melting temperature for the probe on both sides of the junction. Thus, in certain situations, the probe has an increased propensity to cross-hybridize with isoforms containing the same sequence as only one side of the junction, rather than requiring the presence of both sequences for successful hybridization. Introduction of certain mismatches into the probe produces a decreased melting temperature and higher specificity. In preferred embodiments, probes that hybridize to a sequence that encompasses the junction of the fusion genes have 1, 2, 3, 4, 5, or more mismatches. Additional disclosure regarding nanoreporters can be found in International Publication Nos. WO 07/076129 and WO 07/076132, the contents of which are incorporated herein in their entireties. Further, the term nucleic acid probes and nanoreporters can include the rationally designed (*e.g.* synthetic sequences) described in International Publication No. WO 2010/019826, incorporated herein by reference in its entirety.

[76] Examples of various probes and their targets are provided below in Table 1.

Table 1

Gene Fusion	Isoform	Reporter Probe	SEQ ID NO:
BCR-ABL	b2a2	TGGCCGCTGAAGGGCTT CTTCCTTATTGATGGTCA	1
BCR-ABL	b3a2	TGGCCGCTGAAGGGCTT TTGAACCTCTGCTTAAATC	2
BCR-ABL	e1a2	TGGCCGCTGAAGGGCTT CTGCATCTCCATGGAAGG (*)	3
BCR-ABL	e19a2	TGGCCGCTGAAGGGCTT TGACGTCGAAAGCTGCCT (*)	4
BCR-ABL	b3a3	GACCCGGAGCTTTTCAC TTGAACCTCTGCTTAAATC	5
BCR-ABL	b2a3	GACCCGGAGCTTTTCAC CTTCCTTATTGATGGTCA	6
BCR-ABL	e1a3	GACCCGGAGCTTTTCAC CTGCATCTCCATGGAAGG (*)	7
AML-ETO		CTTCACATCCACAGGTGAGTCTGGCATTGTGGAGTGCTTCTCAGTACGAT (+)	8
CBFb-MYH11	e5e12	TCCAGGGCCCGCTTGGACTTCTCCAGCTCATGGAC (+)	9
CBFb-MYH11	e5e8	CGGCCTCGTTAAGCATCCCTGTGACGCTCTCAACTTCATT (+)	10
CBFb-MYH11	e5e7	AGCGTCTGCTTATTCTTGTCTAGGTTTCGCTTGGC (+)	11
E2A-PBX1	e13-insertion-n27-e2	AAAAGGTTCCAGGTGACCGAACACTTTTCAT CACTG	12
E2A-PBX1	e13e2	TCCTCGGATACTCAAAA CACTGTAGGAGTCGGGAG	13
MLL-AF4	e8e7	AGATGTATCATATTGTT CTGATCTGGTGGTGGAG	14
MLL-AF4	e9e5	CATGAATGGGTCATTTC CTTTCTTTTGGTTTTTG	15
MLL-AF4	e9e4	TCATTGGAGTAGGTCTG CTTTCTTTTGGTTTTTG	16
MLL-AF4	e10e5	CATGAATGGGTCATTTC CTAAAGTCCACTCTGAT	17
MLL-AF4	e10e4	TCATTGGAGTAGGTCTG CTAAAGTCCACTCTGAT	18
MLL-AF4	e11e5	CATGAATGGGTCATTTC CTATACATGCCCACTACT (*)	19
MLL-AF4	e11e4	TCATTGGAGTAGGTCTG CTATACATGCCCACTACT (*)	20
PML-RARA	bcr1 (e6e3)	TCTGGGTCTCAATGG CTGACTCCCAAGCGCCACTG (*)	21
PML-RARA	bcr3 (e3e3)	TCTGGGTCTCAATGG CTTTCCACTGGGTGATGCAA (*)	22
PML-RARA	bcr2 (e6partial_e3)	GCTCTGGGTCTCAATGG TTCCTATGACGGGACTCC (*)	23
TEL-AML1	e5e2	TCCCTAGGGCCACCAC CTGCTATTCTACAATGG (*)	24
TEL-AML1	e5e3	CAGAGTGAAGCTTTTCC CTGCTATTCTACCAATGG (*)	25
Gene Fusion	Isoform	Capture Probe	SEQ ID NO:
BCR-ABL	b2a2	GCGGAATGCTGTGGACAGTCTGGAGTTTCACACACGAGTT	26
BCR-ABL	b3a2	CAGTGGCTGAGTGGACGATGACATTCAGAAACCCATAGAGCCCCGGAGAC	27
BCR-ABL	e1a2	CGCCCTCGCCATCGTTGGGCCAGATCTGCCCGGTCTTGCG	28
BCR-ABL	e19a2	TCAGTGCCTGGATGTCCGTGGCCACACCGGACACG	29
BCR-ABL	b3a3	CAGTGGCTGAGTGGACGATGACATTCAGAAACCCA	30
BCR-ABL	b2a3	GCGGAATGCTGTGGACAGTCTGGAGTTTCACACAC	31
BCR-ABL	e1a3	CGCCCTCGCCATCGTTGGGCCAGATCTGCCCGGTCTTGCG	32
AML-ETO		TTTCGAGGTTCTCGGGGCCCATCCACTGTGATTTTGATGGCTCTGTGGTAG	33
CBFb-MYH11	e5e12	CAGTTACTGCCAGCAGCTGTGAAACTCTCACCTCCATTTC	34
CBFb-MYH11	e5e8	CAGTTACTGCCAGCAGCTGTGAAACTCTCACCTCCATTTC	35

CBFb-MYH11	e5e7	CAGTTACTGCCAGCAGCTGTGAAACTCTCACCTCCATTTC	36
E2A-PBX1	e13-insertion-n27-e2	TAGGAGTCGGGAGGCCGAGACAGGTCAGGGAGGGT	37
E2A-PBX1	e13e2	GCCGAGACAGGTCAGGGAGGGTGCCTGGCTGGCTG	38
MLL-AF4	e8e7	GCTGCTTTTCTTGGGCTCACTAGGAGTGGTTTTG	39
MLL-AF4	e9e5	TTTACAGGGATACTTGGGCGGGAGCCACTTTTT	40
MLL-AF4	e9e4	TTTACAGGGATACTTGGGCGGGAGCCACTTTTT	41
MLL-AF4	e10e5	CCTGTGGACTCCATCTGCTGGAATTTTTTGCTTAG	42
MLL-AF4	e10e4	CCTGTGGACTCCATCTGCTGGAATTTTTTGCTTAG	43
MLL-AF4	e11e5	GGCACAGAGAAAGCAAACCACCTGGGTGTTATAG	44
MLL-AF4	e11e4	GGCACAGAGAAAGCAAACCACCTGGGTGTTATAG	45
PML-RARA	bcr1 (e6e3)	GCCACGTGGTTGCTGTGGGCAGGAAGACCTCACTTCCTAT	46
PML-RARA	bcr3 (e3e3)	GAGCTGAGGTCCTGCAGGCGCACCTTGAACTCGTCGAAGC	47
PML-RARA	bcr2 (e6partial_e3)	TGGGGCTAGGCGGTCCATCCAGGTGGGGTGGTGAG	48
TEL-AML1	e5e2	GCATGGCGTGCTCTTCAGGCGGGGAGACAGAGACC	49
TEL-AML1	e5e3	GCATGGCGTGCTCTTCAGGCGGGGAGACAGAGACC	50

[77] Probes in Table 1 marked with (*) are mismatch junction probes that hybridize to the sequence across the gene fusion junction. Each has one, two or three mismatches shown underlined. These mismatches stop the probe from inappropriately hybridizing to a transcript which has only half of the relevant target sequence (for example, the wild-type transcript of one of the fusion genes), by lowering the amount of partial sequence complementarity so that stable hybridization can only occur with the true target. Such mismatches are only necessary in cases where the T_m of the sequence of one exon at the junction is high enough to be stable under standard hybridization conditions, and the sequence cannot simply be shortened because the shorter probe will not retain enough sequence specificity.

[78] Probes in Table 1 marked with (+) are border probes. These probes do not hybridize to a sequence that crosses the gene fusion junction. The target sequence of these reporter probes is adjacent to the target sequence of the capture probes. Each probe only hybridizes to a sequence on one side of the gene fusion junction. The probe pair (capture and reporter) meets at the border of the fusion, with the capture probe sitting in the upstream exon and the reporter probe sitting fully in the downstream exon.

[79] The unmarked sequences in Table 1 are probes with target sequences that cross the gene fusion junction, but do not have mismatches. The sequences of this class of probes are the exact reverse complement of the target sequence. Gaps in the sequence represent the fusion border;

the probe sequence is actually contiguous across this gap. In these probe pairs, the reporter probe spans the fusion junction, and the capture probe sits adjacent in the upstream exon.

[80] The disclosure also describes a panel of probes. These probes include probes for the detection of fusion genes from several gene families from various leukemia subtypes. The panel can contain probes for detection of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more fusion genes. The genes can be from 1, 2, 3, 4, 5, 6, 7 or more gene families. The fusion genes can be present in any leukemia subtype including CML, AML or ALL.

[81] The fusion events, or translocations, usually involve only one of the pair of each chromosome, leaving one intact copy, or “wild-type” copy, of each gene also present in the cell. The panel of probes can also include probes specific for mRNAs produced by the wild-type copies of the relevant genes, allowing the level of expression of the intact version of each gene involved in the translocation to be determined. The panel can include probes specific for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more wild-type mRNAs.

[82] The panel of probes can also include probes specific for mRNAs that are clinically relevant for the detection and diagnosis of leukemia. These markers for leukemia can include WT1, FLT3, BAALC, PRAME, MLLT11, ERG, MN1, RHAMM, HOX11L2, EVI1, N-RAS, RB1 and TP53.

[83] The panel of probes can also include probes specific for housekeeper mRNAs. The results produced from these probes can be used as an internal control to estimate the amount of sample present in the assay. Probes for 1, 2, 3, 4 or more housekeeper mRNAs can be included in the panel.

[84] An example of a panel of probes for the detection and/or diagnosis of leukemia or specific subtypes of leukemia is shown below in Table 2.

Table 2.

Gene Type/Fusion Gene Family	Fusion Gene Isoform	Target Sequence (5'-3')	SEQ ID NO:	Reporter Probe (5'-3')	SEQ ID NO:	Capture Probe (5'-3')	SEQ ID NO:
Housekeeping Genes	GUSB	UCUGUGGCCAACGAGCUGCGUCCACCUAGAAU CUGCUGGCUACUACUUGAAGAUUGGUAUCGCUCA CACCAAUCCUUGGA (SEQ ID NO:)	51	TCCAAGGATTGGT GTGAGCGATCACCA TCCTCAAGTAGTAG CCAGC	52	AGATTCTAGGTGGG ACGAGGCTCGTTG GCCACAGA	53
		CGCCGGCUGUUUAAUUGGCUUCCGCGUGGCCCAU AGUGAUUUUGCAGUGACCCAGCAGCAUCACUGU UUUUGGCGUGUGAAGAUAAACCCAAAGGAUUUG	54	CAATTCCTTGGGTT ATCTTCACAGCCA AGAAACAGTGATGC TGCTGGGT	55	CACTGCAAAAGATCA CTATGGCCAGCGG AAGCGAAGTTAAA CAGCCGGCG	56
	TBP	CUGUGAUGACUUAUUGGUGACUUGUGGAGAU GCAGCGAAUUGUGAAUCCCAACGUAUUAUGCCAUUU CCCUACGCUUGGACCGUACCUUGGGAAGAU	57	GATCTCCAGAACG GTACGCGAGCGTAG AGGAAATGGCATA TACGTGGG	58	ATTTCAGATTGGCT GCATCTCCACAAGT CAGCAATTGAAGTC AATCACAG	59
	ABL1	CUGGGAGACAGGGUGAAGGGAGUGGUUUUAUGA ACUUAACUUAAGAGUCUAAAGAUUUUCUACUGGAU CACUUGUCAAGAUGCGCCUUCUCUGGGGAGAA	60	TTCTCCCCAGAGAG GGCGATCTTTGACA AGTGATCCAGTAGA AATCTTT	61	AGACTCTAAGTTAA GTTTATAAAAAACCA CTCCCTTCAACCTG TCTCCAG	62
	BCR	GGAACGGCACUGUUCACCUUUUAUCUGACCAAACC GCUCUACACGUCAGCACCAUCUCUGCAGCAUCUC UGUAGGCUACCAUUAACAAUAGUACCGGUGC	63	GCACCGGTACATTT GTTAATGGTGAGCC TACAGAGATGCTGC AGAGATGG	64	TGCTGACGTGTAGA GCGGTTTGGTCAGA TAAAGGTGAACAG TGCCGTTC	65
	SOCS2	UCAGAGAGCAAGGCAUCGCGGGUGAAUUCUUGUCU AACAUUCCGAGGUCAGCCAGGCGUCUAAACUUGG AAAGCAGGAUGUAGUUCUGCCAGGCAACUUIU	66	AAAAGTTGCCCTGGC AGAACTACATCTG CTTCCAGGTTAGC AGCTGGC	67	TGACCTCGGGAATG TTAGACAAGATTCA CCCCCGATGCCCTTG CTCTCTGA	68
Leukemia Biomarkers	WT1	AAUUGGAAAAACAGGACGCCUUGGUCUGCAUUC UGAGAGCGUUCAGAGCCGUAUCUGGAAUUGGGUG CUUUGGAUUCACAGGGGGAAAGCUGUAAAGA	69	TCCTTACAGCTTCC CCCTGTGAATCGCA AAGCACCCATTCCA CGATCGG	70	CTCTGGAACGCTCT CAGATATGCAGACC AGGCGTCTCTGGTT TTCCATT	71
	FLT3	AAGAAUCACAAAAGAACUGUGUCAACUAGCAGAGA GUCCAAGCAGAAAGGCGAGAUUGGACUUCUUCAGUG UCCUUCACGGCACUGGAUCCCAUCAAAAGAAC	72	GGTCTTTGTATGGG ATCCAGTGCCGTGA AGGACACTGAAGAA GTCCATCT	73	GCCCTTCTGCTTGG ACTCTCTGCTAGTT GACACAGTTCTTTG TGATTTCT	74
	BAALC	GGUUCUUGAUGAGUGUGGGAUCACGGAUACAG CUCCUUGCCUUCUGGCUUCCUUGAGCCACUGGCU	75	TTAAGGTTGTAAAGC TGGGAGCAGTGGCT	76	GGCAAGGAGCTGA TCATCCGTGATCCC	77
	PRAME						

	CCAGCUUACAACCUUAA		CAGGGAAGGCAGG AG		ACACTCATCAAGA CC	
MLLT1 1	GGAAUAGGACUAGGUUUAUUUACCCAUUGUGAGG GUAGAGAGGCGAGUCUGAGGAGCAGGGAUUGGG AGAAAGGUGGAAAAAUACUCUGAUUCUAAA	78	TTTAAGAATCAGAG TATTTTCCACCCCT TCTCCAAATCCCTG CTCCTCC	79	AGACTCGCTCTCT ACCCTCACAATGGG TAAATAAACCTAGT CCTATTCC	80
ERG	GACCUCAUUAUUGUGGGGCUUUUGUUCUCAC AGGUCAGGUAAGAGAUUGGCCUUCUUGGCGCCA CAUUCAGAAUACCGCAGGCAUUIUGGUAAGG	81	CCTACCAAAATGC CTGCGTGAATTCG ATTGTGGAGCCAA GAAGGCCA	82	TCTCTTACCTGACC CTGTGGAGAACAA AGCCCCACATAAT GATGAGGTC	83
MN1	AGGUGACGAACCAAGGAGCCGUCGACUCGCUUG AAUACAAUUAACCGGCGAGGCCGCCUUCGGGACA UUUUGACAUUUUUCGCCCU	84	AGGGCGAAAAACATG TCAAAATGTCCCGA GGGCGCCTCGCCCG G	85	GTAATTGTATTCCA GCGAGTCGACGGCT CCTTGGTTCTGCAC CCT	86
RHAM M	UUGAAACCGGUAGGGAGUGUAUAAUCCGCAUUCAG UUGUCGAGGAGUGCCAGUCACCUUCAGUUUCUGG AGCUGGCCGUAACAUGUCCUUAUCCUAAAGCGG	87	CGCCTTAGGAAAGG ACATGTTGACGGCC AGCTCCAGAAACTG AAGGTGAC	88	TGGCACTCCTCGAC AACTGAATCGGAT TATCACTCCCTACC GGTTTCAA	89
HOX11	ACCACAUUCCAGCCCAAUCCAGGUACGCACAGA CAGGUUUUCACAUAAUUGCAGCCCAUUCUCCAG AACC AUUUGAGGGUGGGGGGUGUUAUUI	90	AATTAACACCCCC CACCCCTCAAAATGG GTTCTGGAGAAATG GGCTGCAT	91	TTATGTGAAAACT GTCTGTGCGTACCT GGATTGGGCTGGG ATGTGTGT	92
HOX11 L2	GGGCGCUCGCCAAAGUCCCUCAAAUAGCGGACG CGCAGGUCAAGACCUUGGUUCCAAACCCGGAGGAC CAAGUGGCGG	93	CCGCCACTTGGTCC TCCGGTTTGGAAAC CAGGTCTTGACC	94	TGCGCGTCCGTCAT TTTGAGGACTTGG CGAGCGCCGC	95
EV11	GGGCGAGGACUAGGAUUAUGGACCAAAAGGAAGA UCGAAUAGUGUAAAGUUDUGGGCCUUAUGUGGG AGAGCAGAGGUCAAACCCUAGAAAGACCCAGUU	96	AACTGGGGTCTTC AGGTTTGACCTCTG CTCTCCACATAAG GCCCAAAC	97	TTTTCACTACTTC GATCTTCTTTTGG TCCATAATTCCTAGT CCTGCCCC	98
N-RAS	CCUUGGUCCUGACUUCUCCUGGAGGAGAAAGUAUUC CUUUGGUGUCUUCAGUCUCACAGAGAAAGCUCCU GCUACUUCGCCAGCUCUCAGUAGUUAUAGUAC	99	GTACTAACTACTG AGAGCTGGGGAAGT AGCAGGAGCTTCTC TGTGAGAC	100	TGAAGACAGCAAC AGGAATACTTCTCC TCCAGGGAAGTCA GGACCAGGG	101
RB1	CCUAUUCGCCGCUAAAUAACAUUUUGUAAACGCCU UCUUGUCGAGCACCAGAAUUAAGAACAUUAUUAUC UGGACCCUUUCCAGCACACCCUUGCAGAAUGA	102	TCAITCTGCAGGGT GTGCTGAAAAGGG TCCAGATGATATGT TCTAATTC	103	TGGGTGCTCAGACA GAAGGCGTTCACA AAGTGTATTTAGCC GGAGATAGG	104
TP53	CAGGCGUACUCCAGCCACCCUGAAGUCCAAAAG GGUCAGUUAACCCCGCCCAUAAAAACUUAUGU UCAAGACAGAAAGGGCCUGACUCAGAC	105	GTCTGAGTCAGGCC CTTCTGTCTTGAAC ATGAGTITTTATG	106	TAGACTGACCCTTT TTGGACTTCAGGTG GCTGGAGTGAGCCC	107

			GCGGGAGG	TG			
Wild Type Transcripts	PML	GAGGUGGGGCGUCGACGACUUCUCCUAGCUUUUCUGA GCUCCAUUGCGCGCCCUAUCUUGGCCUGCUACAA GCUUG	108	CACAGCTTGTAGCA GGCCAAGATAGGCG GGCGCATGGAG	109	CTCAGAAAAGCTGA GGAAGTGTGCAG CCCCACCTC	110
		CUGGGGCCCAUCUAGGAGUGGCAUCUUUUUUGGU GCCUGAAGGCCAGCUCUGGACCUUCCAGGAAA AGUGCCAG	111	CTGGCACTTTTCTGT GGAAGTCCAGAGC TGGCCTT	112	CAGGGCACCAAAA AAGATGCCACTCT AGATGGGCCCCAG	113
		CAGCCAUGAAGAACCAAGGUUGCAAGAUUUUAUGA CCUCAGGUUUGCGGUCGAAGUGGAAGAGGAAA AGCUUCACUCUGACCAUCACUGUCUUA	114	TGAAGACAGTGATG GTCAGAGTGAAGCT TTTCCCTCTTCCACT TCG	115	ACCGACAAACCTG AGGTCAATTAATCT TGCAACCTGGTTCT TCATGGCTG	116
	ETV6 (TEL)	CGCCACUACUACAAACUAAACAUUAUCAGGAAGG AGCCAGGACAAAGGCUUUUUGUCAGGUUUUAUGAA AAGCCAGAUGAAAUCAUGAGUG	117	CACTCATGATTTCA TCTGGGGTTTTCAT AAACCTGAACAAAA GCCTTTGT	118	CCTGGCTCCTTCTCT GATAATGTTAGTT TGATAGTAGGGCG GATTCAGGTTCCGC TCTCGCACTTGCTG CTCCAAG	119
		CUUGGAGCAGCAAGUGCGAGAGCGGAACCUGAU CCCAAAGCAGCCUGUUGAAACGGCGAGAGAGG AAAAAG	120	CTTTCTCTCTTCTCG CCGTTTCAAAACAGG CTGCTTTGG	121	GATTCAGGTTCCGC TCTCGCACTTGCTG CTCCAAG	122
	PBX1	CGGGAGGAAGCAGGACAUUGGAGACAUUUUACAG CAAAUUAUGACCAUCACAGACAGAGUUUGGAUG AGGCGAGGCCAGAAAACAUGCUUUAACUGC	123	GCAGTTTAAAGCAT GTTTCTGGCCTGC GCCTCATCCAACT CTGGTCTG	124	TGATGGTCATAATT TGCTGTAAAATGTC TCCAATGTCTTGCT TCCTCCCG	125
		CAGUGUUGCCCGAGGUGUCGACUCCUGGCA UCAAGCGAUCCUCCUGCCUUAAGCCUCCAGAGUA CUG	126	CAGTACTCTGGAG GCTAAGCGAGGAGG ATGCGTTG	127	ATGCCAGGAGTTG AGACCAAGCCTGG CAACACTG	128
	CBFB		129	CACCTCTCATCTTCT CCTTGGCTCCAGCA ATCATGTAGTAAAA GATGTGG	130	AATGTCTCTCTGTC TCTGGCTTGGCGAA TTGCCCGTGAATTT TCTAGCAG	131
	MYH11	CUGCUAGAAAAUACCGGGCAAUUCGCCAAGCCA GAGACGAGGACAUUCCACAUUUUACUACAU GAUUGCUGGAGCCAAGGAGAAUGAAGAAUG	132	CTGATCACAGCCAT ATGCACATCTTTA GTGATCCGTCAATCA TCTTCA	133	AACACTTCCGCTGA CCACAAAGCACAA TTTACATGTGTCCA CTCATTTTG	134
	MLL	CAAAUAGAGUGGACACAUUAAUUGUCUUUGU GGUCAGCGGAAGUGUUUGAAGAUAGACGGAUC ACUAAAGAAUGUGCAUAUGGCUGUGAUCAG	135	AGGCATCTTCAGTT TGGTGAGCTTGGCT TTGGCAGGCACITTT CAAGTCTG	136	TTTTTCAAAAGGTC TGCTGTGATAGTC CTCCGGCAGTGGTT TCAGTTCA	137
	AFB1 (AF4)	UGAACUGAAACCACUGCCGGAGGACUUAUCGACAG CAGACCUUUGAAAAAACAGACUUGAAAGUGCCUG CCAAAGCCAAAGCUCACCAACUAGAAGUCCU	138	TGGCGCTGAAGGG CTTCTTCTTATTGA	139	GCGGAATGCTGTGG ACAGTCTGGAGTTT	140
BCR-ABL b2a2	AACUCUGUGUGAAACUCCAGACUGUCCACAGCA UUCCGCUGACCAUCAUAAGGAAGAGCCCUUCA						

	GGGGCA	141	TGGTCA	142	CACACACGAGTT	143
<i>b3a2</i>	GUCUCCGGGCUUAUUGGGUUCUGAAUGUCAUC GUCCACUCAGCACUGGAUUUAAGCAGAGUUCAA AAGCCUUCAGCGCCA		TGGCCGCTGAAGGG CTTTTGAACCTCTGCT TAAATC		CAGTGGCTGAGTGG ACGATGACATTCAG AAACCCATAGAGC CCCGGAGAC	
<i>e1a2</i>	CGCAAGACCGGGCAGAUUGGGCCCAACGAGGCG AGGGCGCUUCCAUUGGAGACGACAGAGGCCUUA GCGGCCA	144	TGGCCGCTGAAGGG CTTCTGCAJCTCCAT GGAAGG	145	CGCCCTCGCCATCG TTGGCCACAGATCTG CCCGTCTTGGC	146
<i>e19a2</i>	CGUGUCCGGUGUGGCCACGGACAUCCAGGCACUG AAGGCAGCCUUCGACGUCAAAGCCUUCAGCGGC CA	147	TGGCCGCTGAAGGG CTTTGACGTGAA ~~A~~ GCTGCCIT	148	TCAGTGCCTGGATG TCCGTGGCCACACC GGACACG	149
<i>b3a3</i>	UGGGUUCUGAAUGUCAUGGUCCACUACGCCACU GGAUUUAAGCAGAGUUCAGUAAAGUAAAGCUCGGG UC	150	GACCCGGAGCTTTT CACTTGAACCTCTGC TTAAATC	151	CAGTGGCTGAGTGG ACGATGACATTCAG AAACCCA	152
<i>b2a3</i>	GUGUGUGAAACUCCAGACUUGUCCACAGCAUUCGG CUGACCAUCAUUAAGGAAGGUGAAAGCUCCGGG UC	153	GACCCGGAGCTTTT CACCTTCTTATTGA TGGTCA	154	GCGGAATGCTGTGG ACAGTCTGGAGTTT CACACAC	155
<i>e1a3</i>	CGAAGACCGGGCAGAUUCUGGGCCCAACGAGGCG AGGGCGCUUCCAUUGGAGACGCGAGGUGAAAGCU CCGGGUC	156	GACCCGGAGCTTTT CACCTGCACTCCA TGAAGG	157	CGCCCTCGCCATCG TTGGCCACAGATCTG CCCGTCTTGGC	158
AML-ETO	CUACCACAGAGCCAUCAAAAUACAGUGGAUGGG CCCCGAGAAACCUUGAAAUUCGUACUGAGAAAGCACU CCACAUGCCAGACUACCUUGUGGAUGUGAAG GAAUUGGAGGUGAGAGUUDUCACAGCUGCUGGCGAG UAAUCUGGUCCAUGAGCUGGAGAGAAUCCAAGCGGG CCUUGA	159	CTTCACATCCACAG GTGAGTCTGGCAJT GTGGAGTGTCTCTC AGTACGAT	160	TTGAGGTCTCTCGG GGCCCATCCACTGT GATTTGATGGCTC TGTGGTAG	161
<i>e5e12</i>	GAAUUGGAGGUGAGAGUUDUCACAGCUGCUGGCGAG UAAUCUGGUCCAUGAGCUGGAGAGAAUCCAAGCGGG CCUUGA	162	TCCAGGGCCGCTT GGACTTCTCCAGCT CATGGAC	163	CAGTTACTGCCAGC AGCTGTGAAACTCT CACCTCCATTTC	164
<i>e5e8</i>	GAAUUGGAGGUGAGAGUUDUCACAGCUGCUGGCGAG UAAUCUGGUCCAUGGCGAAGCUAGACAAAGAAUAAAGC AGACGCU	165	CGCCCTCGTTAAGC ATCCCTGTGACGCT CTCAACTTCATT	166	CAGTTACTGCCAGC AGCTGTGAAACTCT CACCTCCATTTC	167
<i>e5e7</i>	GAAUUGGAGGUGAGAGUUDUCACAGCUGCUGGCGAG UAAUCUGGUCCAUGGCGAAGCUAGACAAAGAAUAAAGC AGACGCU	168	AGCGTCTGCTTATT CTTGCTAGGTTTCG CCTTGGC	169	CAGTTACTGCCAGC AGCTGTGAAACTCT CACCTCCATTTC	170
<i>e13- insertio n-n27- e2</i>	ACCCUCCCUGACCUUGUCUGGCGCCUCCCGACUCCUA CAGUGAU ^U GAAAGUGUUCGGUACACCUUGGAACCUUU U	171	AAAAGTTCCAGGT GACCGAACACTTTC ATCACTG	172	TAGGAGTCGGGAG GCCGAGACAGGTC AGGGAGGGT	173
<i>e13e2</i>	AGCCAGCCAGGCACCCUCCUCCUGACCUUGUCUGGCGC UCCCGACUCCUACAGUGUUUUUGAGUAUCCGAGGA	174	TCCTCGGATACTCA AAACACTGTAGGAG	175	GCCGAGACAGGTC AGGGAGGGTGCCT	176

MLL-AF4	<i>e8e7</i>	CAAAACCAUCCUAGUGAGGCCCAAGAAAAAGCAG CU	177	TCGGGAG	178	GGCTGGCTG	179
	<i>e9e5</i>	AAAAAGUGGUCUCCCGCCCAAGUAUCCCUUAAA ACAAAAACCAAAAGAAAAAGGAAUAGACCCAUUCA UG	180	CATGAATGGGTCAAT TTCTTTTCTTTTGG TTTTTG	181	TTTACAGGGATAC TTGGCGGGGAGC CACTTTT	182
	<i>e9e4</i>	AAAAAGUGGUCUCCCGCCCAAGUAUCCCUUAAA ACAAAAACCAAAAGAAAAAGCAGACCUACUCCAAU GA	183	TCATTTGGAGTAGGT CTGCTTTTCTTTTGG TTTTTG	184	TTTTACAGGGATAC TTGGCGGGGAGC CACTTTT	185
	<i>e10e5</i>	CUAAGCAAAAAUCCAGCAGAGUAGGAGUCCACAG GAUCAGAGUGGACUUAAGGAAUAGACCCAUUCA UG	186	CATGAATGGGTCAAT TTCTTAAAGTCCA CTCTGAT	187	CCTGTGGACTCCAT CTGCTGGAATTTT TGCTTAGC	188
	<i>e10e4</i>	CUAAGCAAAAAUCCAGCAGAGUAGGAGUCCACAG GAUCAGAGUGGACUUAAGCAGACCUACUCCAAU GA	189	TCATTTGGAGTAGGT CTGCTTAAAGTCCA CTCTGAT	190	CCTGTGGACTCCAT CTGCTGGAATTTT TGCTTAGC	191
	<i>e11e5</i>	CUAUAACACCCAGGGUGGUUUGCUUUCUGUGUC CAGUAGUGGGCAUGUAGAGGAAUAGACCCAUUCA UG	192	CATGAATGGGTCAAT TTCTTAAAGTCCA CACTACT	193	GGCACAGAGAAAAG CAAAACCCCTGGG TGTATAG	194
	<i>e11e4</i>	CUAUAACACCCAGGGUGGUUUGCUUUCUGUGUC CAGUAGUGGGCAUGUAGAGCAGACCUACUCCAAU GA	195	TCATTTGGAGTAGGT CTGCTTAAAGTCCA CACTACT	196	GGCACAGAGAAAAG CAAAACCCCTGGG TGTATAG	197
	<i>bcr1</i> (<i>e6e3</i>)	AUAGGAAGUGAGGUCUCCUGCCCAACAGCAACC ACGUGGCCAGUGGCCCGGGGAGGCGCAUUGA GACCCAGA	198	TCTGGGTCTCAATG GCTGACTCCCAAGC GCCACTG	199	GCCACGTGGTTGCT GTTGGCAGGAAG ACCTCACTTCTAT	200
	<i>bcr3</i> (<i>e3e3</i>)	GGCUUCGACGAGUUCACACCCAGGCGCCUGCAGGACC UCAGCUCUUGCAUACCCAGGGGAAAGCCAUUGA GACCCAGA	201	TCTGGGTCTCAATG GCTTTCCACTGGGT GATGCAA	202	GAGCTAGGTCTCTG CAGGCGCACTTGA ACTCGTCGAAGC	203
	<i>bcr2</i> (<i>e6parti</i> <i>al_e3</i>)	CUCACCAACCCACCUUGGAGUAGGACCGCUAGCCCCA GGAGCCCCGUCUAGGAAACCAUUGAGACCCAGAG C	204	GCTCTGGGTCTCAA TGGTTCTCTATGACG GGACTCC	205	TGGGGCTAGGCGGT CCATCCAGGTGGGG TGGTAG	206
TEL-AML1	<i>e5e2</i>	GGUCUCUGUCUCCCGCCUUGAAGAGCAGCGCCCAUG CCCAUUGGGAGAAUAGCAGGUGGUGGCCCUAGGG GA	207	TCCCTAGGGCCAC CACCTGCTATTCTC ACAATGG	208	GCATGGGTGCTCT TCAGGCGGGGAGA CAGAGACC	209
	<i>e5e3</i>	GGUCUCUGUCUCCCGCCUUGAAGAGCAGCGCCCAUG CCCAUUGGGAGAAUAGCAGGGGAAAGCUUCACUC UG	210	CAGAGTGAAGCTTT TCCCTGCTATTCTAC CAATGG	211	GCATGGGTGCTCT TCAGGCGGGGAGA CAGAGACC	212

nCounter® Analysis System overview

[85] The basis of the nCounter® Analysis system is the unique code assigned to each gene to be assayed (International Patent Application No. PCT/US2008/059959 and Geiss *et al.* Nature Biotechnology. 2008. 26(3): 317-325; the contents of which are each incorporated herein by reference in their entireties). The code is composed of an ordered series of colored fluorescent spots which create a unique barcode for each target to be assayed. A pair of probes is designed for each target, a biotinylated capture probe and a reporter probe carrying the fluorescent barcode.

[86] Specific reporter and capture probes are synthesized for each target. Briefly, sequence-specific DNA oligonucleotide probes are attached to code-specific reporter molecules. Capture probes are made by ligating a second sequence-specific DNA oligonucleotide for each target to a universal oligonucleotide containing biotin. Reporter and capture probes are all pooled into a single hybridization mixture, the “probe library”.

[87] The expression levels of all targets are measured in a single multiplexed hybridization reaction. The sample is combined with the probe library, and specific hybridization of the reporter and the capture probes to their target molecules occurs in solution. After hybridization, the tripartite hybridized complexes are purified in a two-step procedure using magnetic beads linked to oligonucleotides complementary to universal sequences present on the capture and reporter probes. This dual purification process allows the hybridization reaction to be driven to completion with a large excess of target-specific probes, as they are ultimately removed, and, thus, do not interfere with binding and imaging of the sample. All post hybridization steps are handled robotically on a custom liquid-handling robot (Prep Station, NanoString Technologies).

[88] Purified reactions are deposited by the Prep Station into individual flow cells of a sample cartridge, bound to a streptavidin-coated surface via the capture probe, electrophoresed to elongate the reporter probes, and immobilized. After processing, the sample cartridge is transferred to a fully automated imaging and data collection device (Digital Analyzer, NanoString Technologies). The expression level of a target is measured by imaging each sample and counting the number of times the code for that target is detected. For each sample, typically 600 fields-of-view (FOV) are imaged (1376 X 1024 pixels) representing approximately 10 mm² of the binding surface. Typical imaging density is 100-1200 counted reporters per field of view depending on the degree of multiplexing, the amount of RNA, and overall target expression

levels. Data is output in simple spreadsheet format listing the number of counts per target, per sample.

[89] In certain embodiments the analysis system used herein also used a reference sample as a control. The reference samples are known quantities of nucleic acid fragments with sequences that correspond to one or more fusion genes. For example, a reference sample with a sequence corresponding to a specific fusion gene is used with a nanoreporter with a target-specific probe that specifically binds to a sequence on the same fusion gene. When multiple nanoreporters are used for multiple fusion genes, the reference sample may contain multiple target nucleic acids which correspond to the sequences that the target specific probes of the nanoreporters specifically bind to.

Kits

[90] The present disclosure also describes kits useful for detecting and diagnosing leukemia. These kits comprise a set of probes described above. For example, the kit may include any number of probes described in Table 2, above. The kit may further comprise a computer readable medium.

[91] In another embodiment, the kit includes probes that may be provided in a lyophilized or reconstituted form, or may be provided as a set of nucleotide sequences. In one embodiment, the probes are provided in a microplate format, where each probe set occupies a well (or multiple wells, as in the case of replicates) in the microplate. The kit may further comprise reagents and instructions sufficient for the detection of the various target sequences described above.

EXAMPLE

[92] **Example 1. Functional performance of fusion probes showing specificity for fusion gene isoforms on synthetic gene fragments.**

[93] A multiplexed collection of nanoreporters containing probes to all the isoforms of BCR-ABL, E2A-PBX1, TEL-AML1 and PML-RARA listed in Table 1 was used to detect synthetic transcripts of sequences corresponding with each of these gene fusions. An nCounter® leukemia fusion gene expression assay was performed as described below, and some of the resulting counts for each probe are shown in log2 scale in Figure 1. Cross-hybridization for each individual probe to non-specific targets was below 1%.

[94] The leukemia fusion gene expression assay was run on the nCounter® Analysis system (NanoString). Each sample was hybridized to the multiplexed nanoreporters in solution, followed by post-hybridization sample processing on the nCounter® Prep Station and data collection on the nCounter® Digital Analyzer.

[95] Hybridization Protocol

[96] Each hybridization reaction contained the following components: 10 µL Reporter CodeSet, 10 µL hybridization buffer, 5 µL synthetic RNA target to a final concentration of 1 pM, and 5 µL Capture ProbeSet.

[97] Aliquots of both the Reporter CodeSet and Capture ProbeSet reagent were removed from the freezer and thawed on ice. The aliquots were inverted several times to mix well. The reagent was briefly spun down at <1000 rpm.

[98] A master mix was created containing 130 µL of the Reporter CodeSet and 130 µL of hybridization buffer by adding the hybridization buffer to the tube containing the Reporter CodeSet. The master mix was inverted to mix and spun down, and 20 µL of master mix was added to each of 12 tubes.

[99] Samples of synthetic target transcripts were denatured 85°C for 5 minutes and quick-cooled on ice before being added to each tube and mixed. Targets were added to a final concentration of 1 pM.

[100] The thermocycler was pre-heated to 65°C. 5 µL of Capture ProbeSet were added to each tube and mixed well before placing immediately at 65°C. Hybridization assays were incubated at 65°C overnight.

[101] Hybridizations were left at 65°C until ready for processing. Once removed from 65°C, the assay proceeded immediately to post-hybridization processing on the nCounter® Prep Station.

[102] Post-Hybridization Processing and Data Collection

[103] After hybridization, an automated protocol on the nCounter® Prep Station was used to wash away excess probes and bind the captured probe/target complexes onto a cartridge, and then stretch and immobilize the captured nanoreporters in preparation for imaging. The purification procedure involved a two-step magnetic beadbased affinity purification. Magnetic beads derivatized with short nucleic acid sequences that were complementary to the Capture Probes and the Reporter Probes were used sequentially. First, the hybridization mixture

containing target/probe complexes was allowed to bind to magnetic beads complementary to sequences on the Capture Probe. Wash steps were performed to remove excess Reporter Probes. After washing, the Capture Probes and target/probe complexes were eluted off the beads and hybridized to magnetic beads complementary to sequences on the Reporter Probe. An additional wash was performed to remove excess Capture Probes. Finally, the purified Capture Probe/target/Reporter Probe complexes were eluted off the beads and the captured nanoreporters were bound, stretched and immobilized on a cartridge for data collection.

[104] Automated data collection was carried out in the nCounter® Digital Analyzer. Digital images of 600 fields of view of the bound nanoreporters were processed and the counts for each nanoreporter barcode were tabulated in a comma separated value (CSV) format.

[105] Example 2. Leukemia Fusion Gene Panel Tested on total RNA isolated from cell lines.

[106] Figures 2A-F show results from a Leukemia Fusion Gene Panel tested on total RNA isolated from 5 cell lines. The assay was performed as described in Example 1 with 100ng of input RNA. Probes included in the CodeSet identified their respective targets expressed in the different cell lines: the BCR-ABL e1a2 isoform expressed in cell line SUP B13 (A), the BCR-ABL b3a2 isoform expressed in cell line K562 (B), the PML-RARA bcr1 isoform expressed in cell line NB4 (C), the AML-ETO fusion gene transcript expressed in the Kasumi cell line (D), the BCR-ABL b2a2 isoform expressed in cell line KCL-22 (E) and the MLL-AF4 e9e5 isoform expressed in cell line MV4-11 (F).

OTHER EMBODIMENTS

[107] While the disclosure has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the disclosure, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

[108] The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. All United States patents and published or unpublished United States patent applications cited herein are incorporated by reference. All published foreign patents and patent applications cited herein are hereby incorporated by reference. Genbank and NCBI submissions indicated by accession number cited herein are hereby incorporated by reference. All other published references, documents, manuscripts and scientific literature cited

herein are hereby incorporated by reference.

[109] While this disclosure has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the disclosure encompassed by the appended claims.

CLAIMS

What is claimed is:

1. A composition for the diagnosis of leukemia or the identification of a subtype of leukemia comprising one or more target-specific nucleic acid probes wherein each of the one or more target-specific probes comprises
 - (i) a target-specific region that specifically binds to one target nucleic acid analyte; and
 - (ii) a region to which is attached a plurality of label monomers that create a unique label for each target-specific probe, said label having a detectable signal that distinguishes one target-specific probe which binds to a first target nucleic acid from another target-specific probe that binds to a different second target nucleic acid,wherein one or more targets of the one or more target-specific probes comprise a fusion gene or mRNA transcribed from said fusion gene and wherein the fusion gene is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia.
2. The composition of claim 1, wherein the fusion gene is selected from the group consisting of *BCR-ABL*, *AML-ETO*, *CBFB-MYH11*, *E2A-PBX1*, *MLL-AF4*, *PML-RARA*, or *TEL-AML1*.
3. The composition of claim 1, wherein the leukemia is selected from the group consisting of chronic myelogenous leukemia, acute myeloid leukemia and acute lymphoblastic leukemia.
4. The composition of claim 1, wherein at least one of the one or more target-specific probes has a target that is the sequence of the junction of the two genes that are fused.
5. The composition of claim 1, wherein at least one of the one or more target-specific probes comprises a target-specific region with one or more mismatches to the target of the target-specific region.
6. The composition of claim 1, wherein each of said label monomers are selected from the group consisting of a fluorochrome moiety, a fluorescent moiety, a dye moiety or a chemiluminescent moiety.

7. The composition of claim 6, wherein each of said label monomers comprise a fluorescent moiety.
8. The composition of claim 1, wherein the unique label for each target-specific probe is composed of 4 label monomers.
9. The composition of claim 1, wherein the unique label for each target-specific probe is composed of 5 label monomers.
10. A composition for the diagnosis of leukemia comprising a probe pair comprising a first probe and a second probe, said first probe comprising a complex comprising:
- (a) a first molecule, comprising:
 - (i) a first label attachment region to which are attached one or more label monomers that emit light constituting a first signal;
 - (ii) a second label attachment region, which is non-overlapping with the first label attachment region, to which are attached one or more label monomers that emit light constituting a second signal; and
 - (b) a first target-specific sequence attached to the first molecule,
- said second probe comprising a second molecule, comprising
- (i) a second target-specific sequence; and
 - (ii) an affinity tag
- wherein the first target-specific sequence and the second target-specific sequence bind to different regions of the same target molecule, wherein the target molecule is a fusion gene or mRNA transcribed from said fusion gene, wherein the fusion gene is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia and wherein when said probe pair is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitute at least part of a code that identifies the target molecule.

11. The composition of claim 10, wherein the fusion gene comprises the fusion of at least a portion of a first gene and at least a portion of a second gene.
12. The composition of claim 11, wherein the first target-specific sequence hybridizes to a portion of the first gene and the second target-specific sequence hybridizes to a portion of the second gene.
13. The composition of claim 12, wherein the first target-specific sequence hybridizes to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene.
14. The composition of claim 11, wherein the first target-specific sequence hybridizes to a portion of the first gene and a portion of the second gene.
15. The composition of claim 14, wherein the first target-specific sequence hybridizes to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene.
16. The composition of claim 14, wherein at least one of the target-specific sequences comprises a region with one or more mismatches to the target of the target-specific sequence.
17. The composition of claim 11, wherein the second target-specific sequence hybridizes to a portion of the first gene and a portion of the second gene.
18. The composition of claim 17, wherein the first target-specific sequence hybridizes to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene.
19. The composition of claim 17, wherein at least one of the target-specific sequences comprises a region with one or more mismatches to the target of the target-specific sequence.

20. The composition of claim 10, wherein the leukemia is selected from the group consisting of chronic myelogenous leukemia, acute myeloid leukemia and acute lymphoblastic leukemia.
21. The composition of claim 10, wherein the fusion gene is selected from the group consisting of *BCR-ABL*, *AML-ETO*, *CBFB-MYH11*, *E2A-PBX1*, *MLL-AF4*, *PML-RARA*, or *TEL-AML1*.
22. The composition of claim 10, wherein each of said label monomers are selected from the group consisting of a fluorochrome moiety, a fluorescent moiety, a dye moiety or a chemiluminescent moiety.
23. The composition of claim 22, wherein each of said label monomers comprise a fluorescent moiety.
24. The composition of claim 22, wherein the unique label for each target-specific probe is composed of 4 label monomers.
25. The composition of claim 22, wherein the unique label for each target-specific probe is composed of 5 label monomers.
26. A method for diagnosing leukemia, a specific subtype of leukemia or determining a genetic aberration underlying leukemia in a subject comprising
- (a) providing a sample from the subject; and
 - (b) contacting the sample with one or more target-specific probes wherein each of the one or more target-specific probes comprise
 - (i) a target-specific region that binds to one target nucleic acid analyte; and
 - (ii) a region to which is attached a plurality of label monomers that create a unique label for each target-specific probe, said label having a detectable signal that distinguishes one target-specific probe which binds to a first target nucleic acid from another target-specific probe that binds to a different second target nucleic acid,

wherein one or more targets of the one or more target-specific probes comprise a fusion gene or mRNA transcribed from said fusion gene that is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia and wherein when the fusion gene is detected the subject is diagnosed with leukemia, a specific subtype of leukemia or the genetic aberration underlying leukemia is determined.

27. The method of claim 26, wherein fusion gene is selected from the group consisting of *BCR-ABL*, *AML-ETO*, *CBFB-MYH11*, *E2A-PBX1*, *MLL-AF4*, *PML-RARA*, or *TEL-AML1*.
28. The method of claim 26, wherein the leukemia is selected from the group consisting of chronic myelogenous leukemia, acute myeloid leukemia and acute lymphoblastic leukemia.
29. The method of claim 26, wherein at least one of the one or more target-specific probes has a target that is the sequence of the junction of the two genes that are fused.
30. The method of claim 26, wherein at least one of the one or more target-specific probes comprises a target-specific region with one or more mismatches to the target of the target-specific region.
31. The method of claim 26, wherein each of said label monomers are selected from the group consisting of a fluorochrome moiety, a fluorescent moiety, a dye moiety or a chemiluminescent moiety.
32. The method of claim 31, wherein each of said label monomers comprise a fluorescent moiety.
33. The method of claim 26, wherein the unique label for each target-specific probe is composed of 4 label monomers.
34. The method of claim 26, wherein the unique label for each target-specific probe is composed of 5 label monomers.

35. A method for diagnosing leukemia, a specific subtype of leukemia or determining a genetic aberration underlying leukemia in a subject comprising:
- (a) providing a sample from the subject; and
 - (b) contacting the sample with a probe pair comprising a first probe and a second probe, said first probe being a complex comprising:
 - (i) a first molecule, comprising:
 - (1) a first label attachment region to which are attached one or more label monomers that emit light constituting a first signal;
 - (2) a second label attachment region, which is non-overlapping with the first label attachment region, to which are attached one or more label monomers that emit light constituting a second signal; and
 - (ii) a first target-specific sequence attached to the first molecule,said second probe comprising a second molecule, comprising
 - (1) a second target-specific sequence; and
 - (2) an affinity tagwherein the first target-specific sequence and the second target-specific sequence bind to different regions of the same target molecule, wherein the target molecule is a fusion gene or mRNA transcribed from said fusion gene, wherein the fusion gene is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia and wherein when said probe pair is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitute at least part of a code that identifies the target molecule and wherein when the fusion gene is detected the subject is diagnosed with leukemia, a specific subtype of leukemia or the genetic aberration underlying leukemia is determined.
36. The method of claim 35, wherein the fusion gene comprises the fusion of at least a portion of a first gene and at least a portion of a second gene.
37. The method of claim 35, wherein the first target-specific sequence hybridizes to a portion of the first gene and the second target-specific sequence hybridizes to a portion of the second gene.

38. The method of claim 37, wherein the first target-specific sequence hybridizes to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene.
39. The composition of claim 35, wherein the first target-specific sequence hybridizes to a portion of the first gene and a portion of the second gene.
40. The method of claim 39, wherein the first target-specific sequence hybridizes to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene.
41. The method of claim 39, wherein at least one of the target-specific sequences comprises a region with one or more mismatches to the target of the target-specific sequence.
42. The method of claim 35, wherein the second target-specific sequence hybridizes to a portion of the first gene and a portion of the second gene.
43. The method of claim 42, wherein the first target-specific sequence hybridizes to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene.
44. The method of claim 42, wherein at least one of the target-specific sequences comprises a region with one or more mismatches to the target of the target-specific sequence.
45. The method of claim 35, wherein the leukemia is selected from the group consisting of chronic myelogenous leukemia, acute myeloid leukemia and acute lymphoblastic leukemia.
46. The method of claim 35, wherein the fusion gene is selected from the group consisting of *BCR-ABL*, *AML-ETO*, *CBFB-MYH11*, *E2A-PBX1*, *MLL-AF4*, *PML-RARA*, or *TEL-AML1*.

47. The method of claim 35, wherein each of said label monomers are selected from the group consisting of a fluorochrome moiety, a fluorescent moiety, a dye moiety or a chemiluminescent moiety.
48. The method of claim 47, wherein each of said label monomers comprise a fluorescent moiety.
49. The method of claim 35, wherein the unique label for each target-specific probe is composed of 4 label monomers.
50. The method of claim 35, wherein the unique label for each target-specific probe is composed of 5 label monomers.
51. A kit comprising one or more reporter probes and one or more capture probes, wherein the reporter probe and the capture probe each bind specifically to a fusion gene that is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia.
52. The kit of claim 51, wherein the fusion gene is selected from the group consisting of *BCR-ABL*, *AML-ETO*, *CBFB-MYH11*, *E2A-PBX1*, *MLL-AF4*, *PML-RARA*, or *TEL-AML1*.
53. The kit of claim 51, comprising one or more reporter probes and one or more capture probes from Table 2.

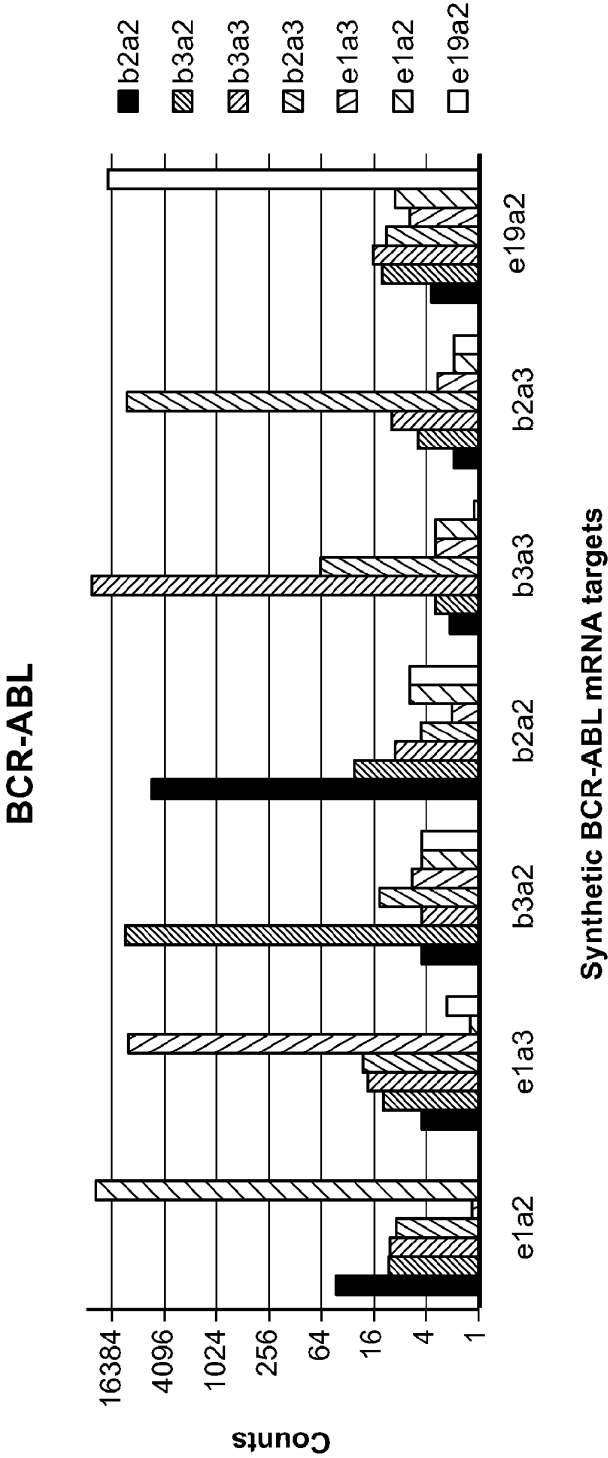


FIG. 1A

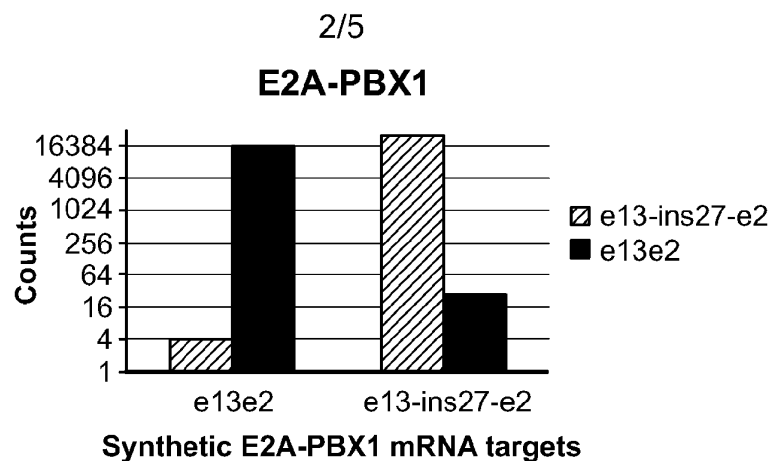


FIG. 1B

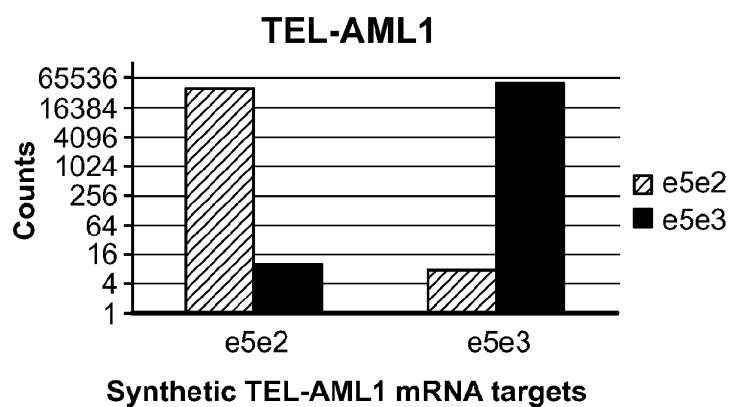


FIG. 1C

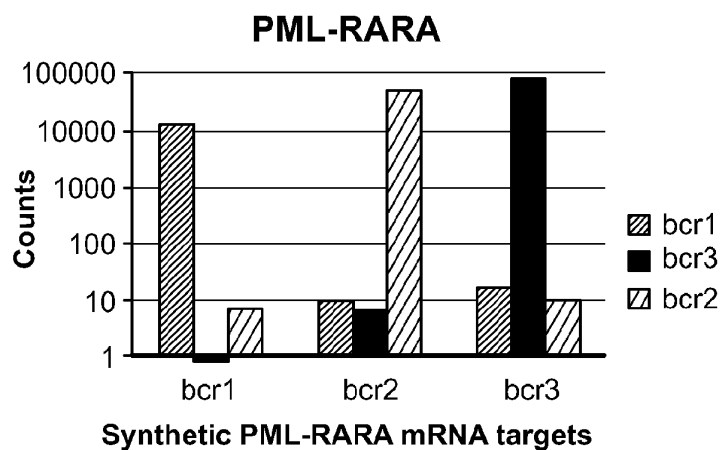


FIG. 1D

SUBSTITUTE SHEET (RULE 26)

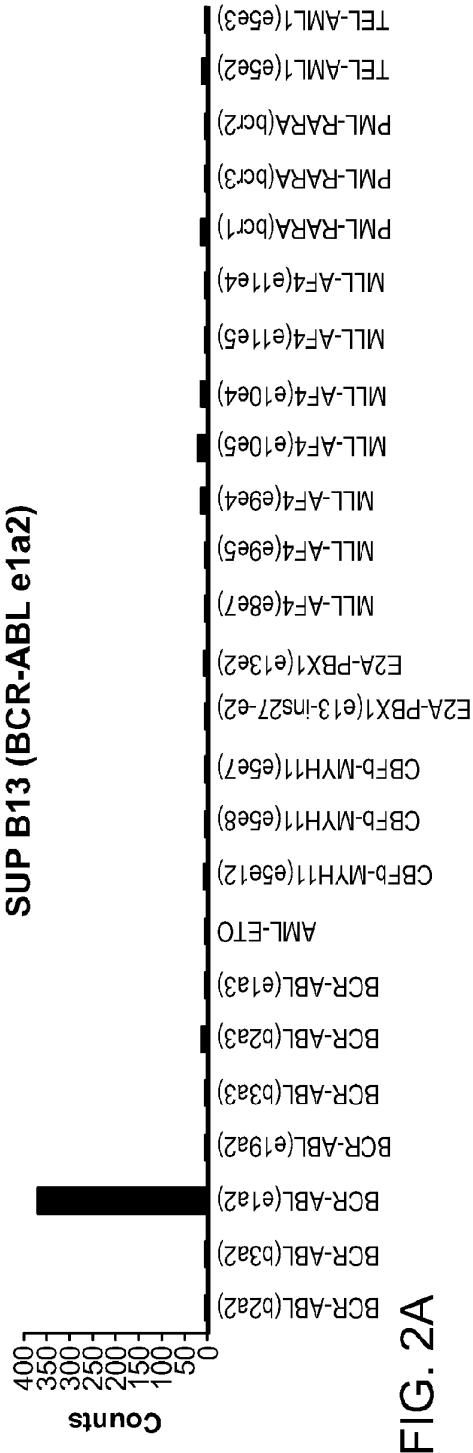


FIG. 2A

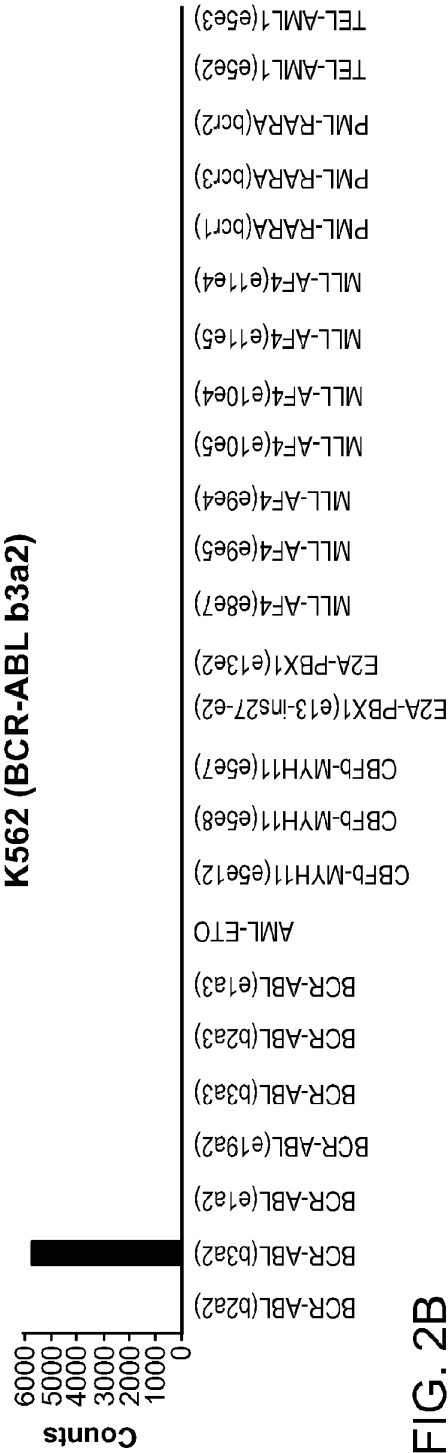
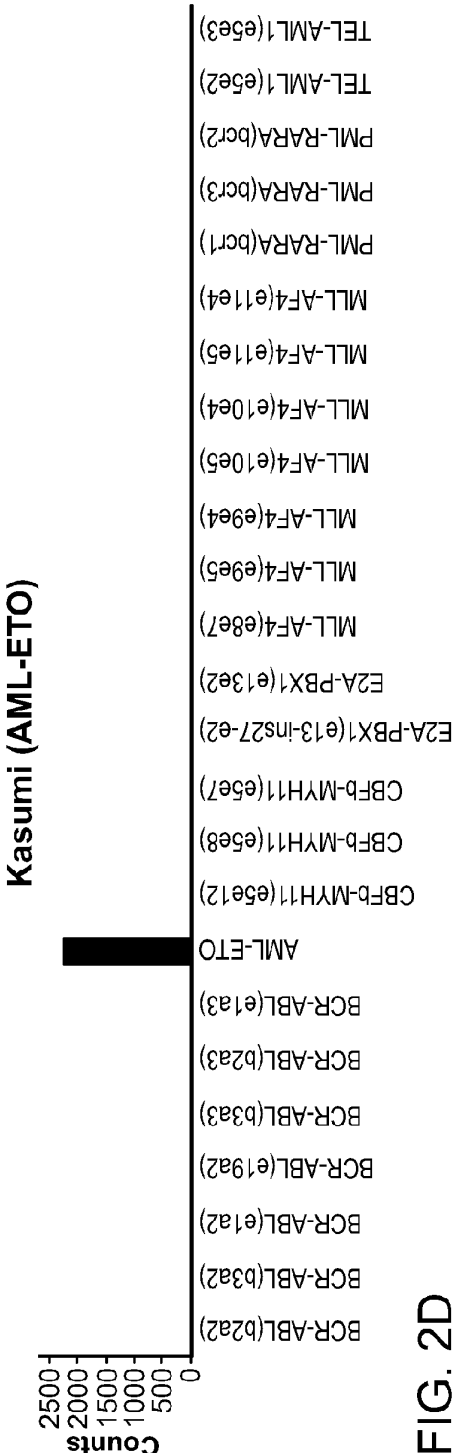
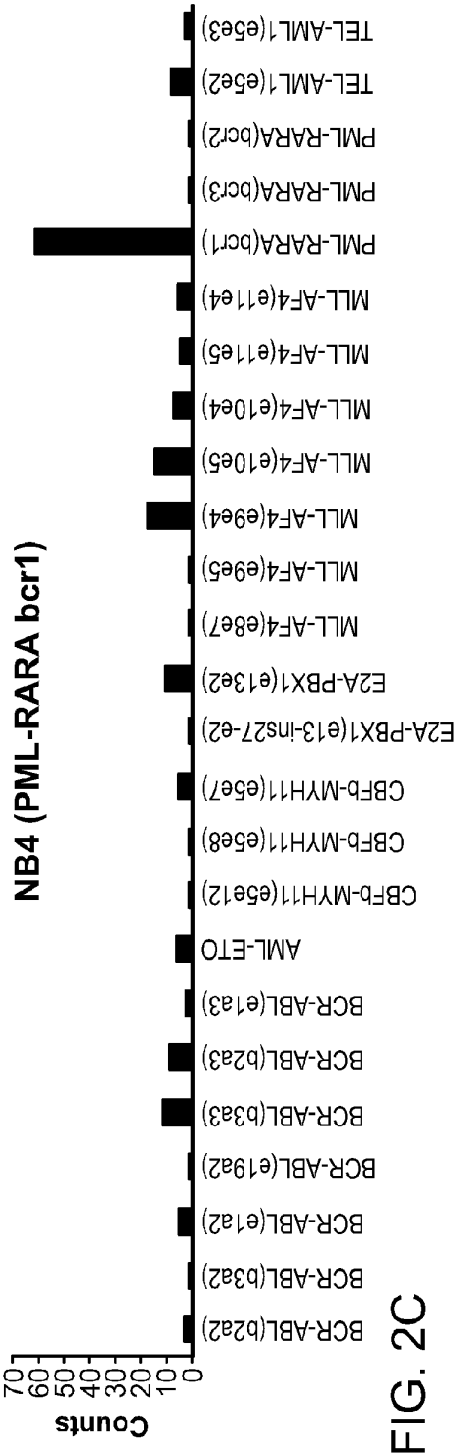
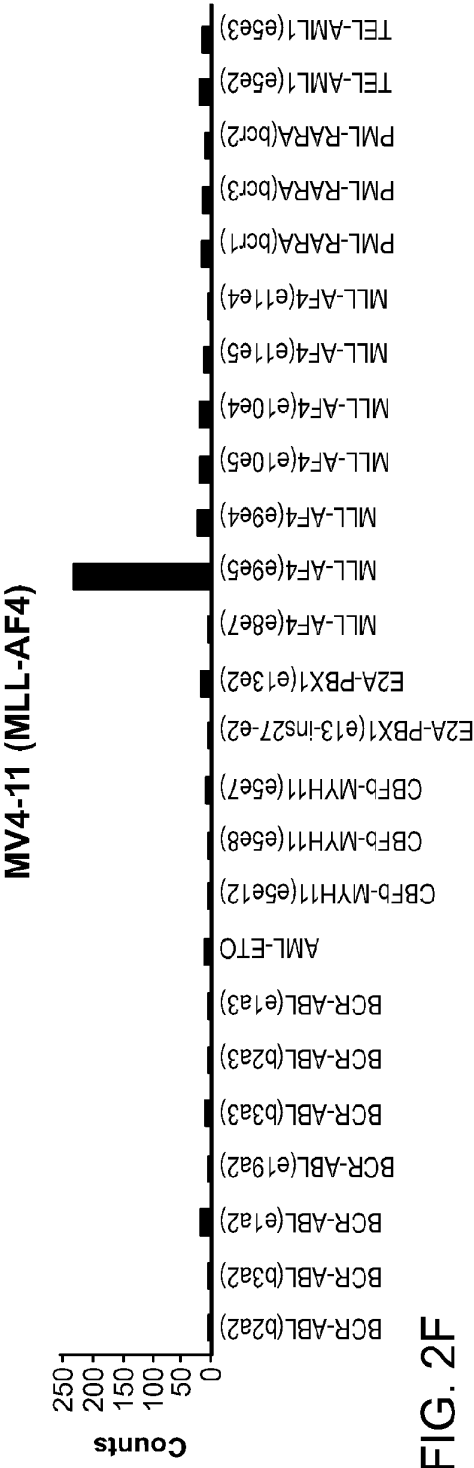
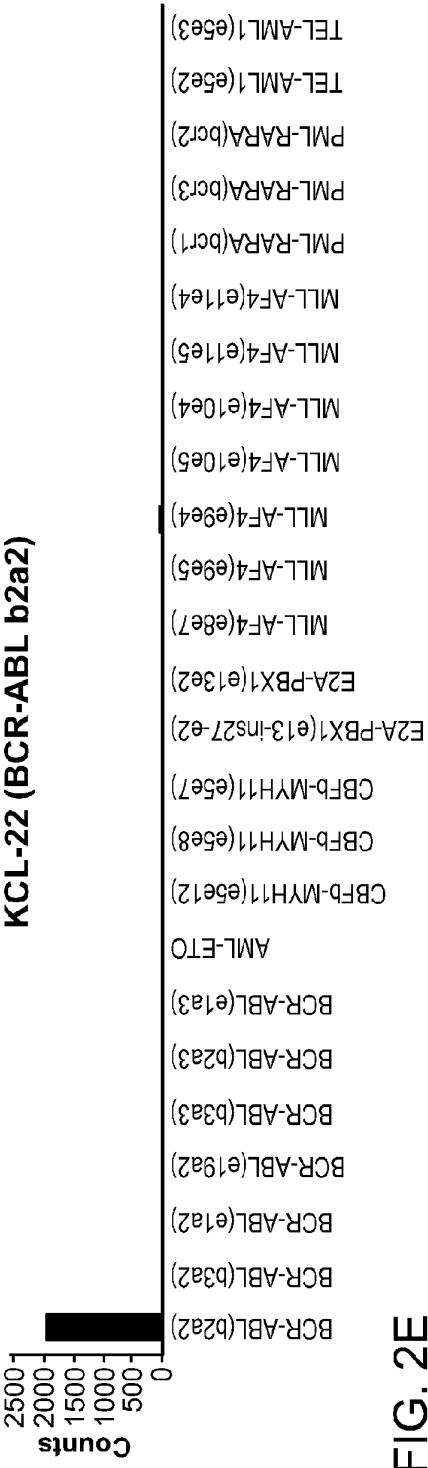


FIG. 2B

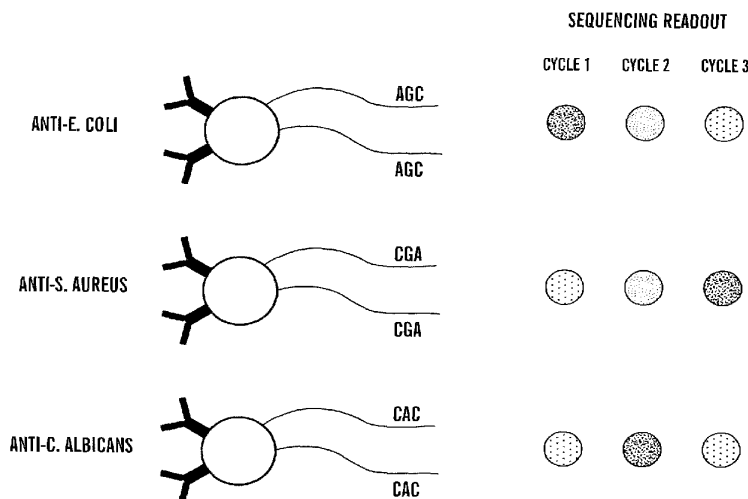






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(54) **Title:** COMPOSITIONS AND METHODS FOR ANALYTE DETECTION



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

COMPOSITIONS AND METHODS FOR ANALYTE DETECTION

CROSS REFERENCE TO RELATED APPLICATIONS

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

GOVERNMENT SUPPORT

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

TECHNICAL FIELD OF THE DISCLOSURE

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

BACKGROUND

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

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

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

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

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

SUMMARY

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

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

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

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

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

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

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

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

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

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

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

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

subsequence of the detection reagents.

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

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

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

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

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

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

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

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

glycol, crosslinker, or combination thereof.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

BRIEF DESCRIPTION OF THE DRAWINGS

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

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

[0043] **Fig. 3** shows exemplary forms of a nucleic acid label of the detection reagent according to one or more embodiments described herein.

[0044] **Figs. 4A and 4B** shows two exemplary configurations of probe reagents and nucleic acid labels on particles, in accordance with one or more embodiments described herein.

[0045] **Fig. 5** shows one embodiment of the detection reagents for an exemplary hybridization-based readout method, in accordance with one or more embodiments described herein. Pathogen-specific antibodies (e.g., anti-*C. albicans*) are individually conjugated to a nanoparticle comprising at least one nucleic acid label as described herein. In accordance with one or more embodiments, the readout of the nucleic acid label can be determined by hybridizing it with a small number of, e.g., fluorescently-labeled decoder probes, imaging, and then advancing to the next set of decoder probes. In order to allow the SeqTag to be read out quickly and without the use of enzymes or chemical reactions, the DNA oligonucleotide (SeqTag) is designed to include several hybridization sites, each corresponding to a particular readout step. At each readout step, the sample is subjected to a mixture of fluorescently labeled DNA probes that could potentially bind that step's hybridization site. Each of the sites, however, is designed

to bind only one of these probes, thus revealing the SeqTag's identifying code.

[0046] **Figs. 6A-6B** show the readout images of DYNABEADS® beads (1 μ m in size) localized on a sample using an exemplary hybridization-based detection method. Each bead was conjugated to one of 6 nucleic acid labels, which in turn hybridized with a different set of decoder probes conjugated to either a green, red or blank optical label during each readout stage (**Fig. 6A**: Readout stage 1; **Fig. 6B**: Readout stage 2).

[0047] **Fig. 7** is a set of images showing that each of the detection molecule constructs (SeqTag labels) properly stained the yeast and fluoresced in accordance with three pre-determined sets of decoder probes. SeqTag labels and oligonucleotide displacers were applied as follow:

- Step 1: Set 1 Probes only;
- Step 2: Set 1 Displacement oligonucleotides and Set 2 Probes;
- Step 3: Set 2 Displacement oligonucleotides and Set 3 Probes; and
- Step 4: Set 3 Displacement oligonucleotides.

SeqTag Label and corresponding colors were as follow:

- Set 1: SeqTag 1 - Green, SeqTag 2 - Red, and SeqTag 3 - Blue;
- Set 2: SeqTag 4 - Green, SeqTag 5 - Red, and SeqTag 6 - Blue;
- Set 3: SeqTag 7 - Green and SeqTag 8 - Red.

[0048] **Fig. 8** shows SeqTag readout by a displacement-hybridization experiment according to an embodiment of the method described herein. SeqTag DNA labels were incubated on a streptavidin-coated microarray. This microarray was then exposed to a sequence of fluorescently labeled detection probes and displacement oligonucleotides, as per displacement-hybridization readout. Imaging the array after each readout step demonstrated fluorescence corresponding to the expected pattern (shown next to each image).

[0049] **Fig. 9** is a schematic representation of displacement hybridization according to an embodiment of the method. As the readout progresses, fluorescence from prior readout steps needst be removed so as not to obstruct the current step's signal. As illustrated in **Fig. 9**, this can be accomplished using a displacement-hybridization method: each of the SeqTag's hybridization sites can be preceded by a short "toehold" sequence. During each readout step, the sample is subjected to a mixture of "displacer" DNA oligonucleotides. One of these displacers is

complementary to the preceding step's hybridization site and, with the help of the toehold, is capable of displacing the fluorescent probe that was bound there.

[0050] **Fig. 10** is a schematic representation of a probe reagent according to an embodiment described herein. Shown is an oligonucleotide-antibody-streptavidin construct. A convenient and effective way to SeqTag-label antibodies is through a streptavidin bridge. The antibody is biotinylated and the probe DNA oligonucleotides (SeqTag) are synthesized with a 5'-biotin modification. Then, by taking advantage of streptavidin's native tetrameric form, three DNA strands are bound to each antibody. As opposed to chemical conjugation methods, which can harm the antibody, this method proves gentle enough to preserve antibody function. As shown a single streptavidin molecule (2) is bound to three of the same DNA SeqTags (1) and a single antibody (3).

[0051] **Fig. 11** is a schematic representation of detection of an analyte by the SeqTag labeled antibody shown in **Fig. 10**.

[0052] **Fig. 12** is a schematic representation of detection reagents for different analytes. As shown, different infectious agent (analytes) can each be assigned their own SeqTag code to enable multiplexed detection according to an embodiment described herein.

[0053] **Fig. 13** is a schematic representation of detection reagents for SeqTagged Fluorescence *in situ* hybridization (FISH). FISH permits microbes to be identified based on their ribosomal RNA sequence. In the case of SeqTagged FISH, the FISH probe and its SeqTag can be synthesized as a single DNA oligonucleotides as shown. Sequences shown are, from top to bottom, SEQ ID NO: 1 (5'-CCTACACACCAGCGTGCC-3', probe for *K. pneumonia*); SEQ ID NO: 2 (5'-CCGCACTTTCATCTTCCG-3', probe for *H. influenza*); and SEQ ID NO: 3 (GCCAAGGCTTATACTCGC, probe for *C. albicans*).

DETAILED DESCRIPTION OF THE INVENTION

[0054] Described herein are methods, detection reagents (or detection molecules as used interchangeably herein) and kits for detecting a plurality of analytes in a sample. In accordance with embodiments of various aspects described herein, a probe reagent (e.g., antibody or aptamers) can be directly or indirectly labeled with a nucleic acid label. The nucleic acid information present on the nucleic acid label can then be decoded and/or detected in a temporally-sequential manner. The detection reagents and methods described herein significantly

increase the number of different probes (and corresponding analytes) that can be simultaneously detected in a multiplex assay, as compared to an traditional assay where each probe is labeled with only fluorescent labels or quantum dots, and thus multiplexing is limited by the number of available and practically usable colors. Furthermore, because the detection reagents described herein are detected and/or imaged in a temporal series of steps, the number of probes (and corresponding analytes) that can be detected in a multiplex assay grows multiplicatively with the number of detection steps in a time series and the number of optical labels being used. By way of example only, 3 set of images in which 4 distinct optical labels are used can encode $4 \times 4 \times 4 = 64$ distinct probe reagents (e.g., antibodies).

Methods of detecting a plurality of analytes in a sample

[0055] One aspect of the inventions provides the methods for detecting a plurality of analytes in a sample, using the detection reagents described herein. The method includes (a) contacting the sample with a composition comprising a plurality of detection reagents (which will be described in detail later), wherein each subpopulation of the detection reagents targets at least one different analyte; and (b) detecting in a temporally-sequential manner said plurality of the pre-determined subsequences of said detection reagents, wherein said detection of the subsequences each generates a signal signature corresponding to said subsequence, and wherein a temporal order of the signal signatures corresponding to said plurality of the subsequences of said detection reagent identifies a subpopulation of the detection reagents. In some embodiments, the signal signature is a temporal signature. In some embodiments, the signal signature can further comprise a spatial signature. A non-limiting example of a spatial signature includes spatial location of a signal signature.

[0056] In some embodiments, the temporal order of the signal signatures corresponding to the plurality of the subsequences of the detection reagent can be unique for each subpopulation of the detection reagents. In some embodiments, at least two or more signal signatures can be used to identify the same subpopulation of the detection reagents.

[0057] In some embodiments, a detection reagent described herein can target at least two (e.g., at least two, at least three or more) distinct analytes. In some embodiments, a first subpopulation of the detection reagents can target at least one analyte different from that of a second subpopulation of the detection reagents. By way of example only, a first subpopulation of

the detection reagents can target at least analyte A and analyte B, whereas a second subpopulation of the detection reagents can target at least analyte B and analyte C. The readout of these detection reagents can be distinct but overlapping. Thus, different analytes can be identified by sampling them combinatorially and determining which one binds.

[0058] As used herein, the term “temporal order of the signal signatures” refers to a sequence of signal signatures determined in a temporally-sequential manner, i.e., the sequence of signal signatures is progressed through by a number of active operations performed in a temporally-sequential manner, e.g., using a different set of decoding reagents or decoder probes in each active operation. In some embodiments, using a set of decoder probes in each active operation can yield one signal signature corresponding to one subsequence of the detection reagents.

[0059] The composition comprising a plurality of detection reagents can exist in any format. In some embodiments, the composition comprising a plurality of detection reagents can be in a form of a solution or suspension comprising the detection reagents. In such embodiments, the composition can further comprise at least one agent. For example, without wishing to be bound, the agent can be a blocking buffer, a surfactant, unconjugated probe reagents, a stabilizer, an enzyme inhibitor, or any combinations thereof. In some embodiments where the compositions are administered *in vivo*, the composition can further comprise a pharmaceutically-acceptable carrier. In other embodiments, the composition comprising a plurality of detection reagents can be contained or immobilized in a device (e.g., a syringe, or a microfluidic device) or an assay or reaction vessel (e.g., solid supports such as vials, and multi-well plates).

[0060] As used therein, the term “contacting” refers to any suitable means for delivering, or exposing, a sample to a plurality of the detection reagents described herein. In some embodiments, the term “contacting” refers to adding the detection reagents (e.g., suspended in a solution) directly to the sample. In some embodiments, the term “contacting” can further comprise mixing the sample with the detection reagents by any means known in the art (e.g., vortexing, pipetting, and/or agitating). In some embodiments, the term “contacting” can further comprise incubating the sample together with the detection reagents for a sufficient amount of time, e.g., to allow binding of the probe reagents to the target analytes. The contact time can be of any length, depending on the binding affinities and/or concentrations of the probe reagents and/or the analytes, concentrations of the detection reagents, and/or incubation condition (e.g., temperature). For example, the contact time can be reduced if the sample and detection reagents

are incubated at a higher temperature. In some embodiments, the contact time between the sample and the detection reagents can be at least about 30 seconds, at least about 1 minute, at least about 5 minutes, at least about 10 minutes, at least about 15 minutes, at least about 30 minutes, at least about 1 hour, at least about 2 hours, at least about 3 hours, at least about 4 hours, at least about 6 hours, at least about 8 hours, at least about 10 hours, at least about 12 hours, at least about 24 hours, at least about 48 hours or longer. One of skill in the art can adjust the contact time accordingly.

[0061] For in vivo applications, the term “contacting” can refer to administering the detection reagents to a subject, e.g., by oral administration or by injection.

[0062] The sample can be contacted with at least one kind of the detection reagents. In some embodiments, the sample can be contacted with at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100 or more different kinds of the detection reagents. In some embodiments, the sample can be contacted with at least 100, at least 500, at least 1000, at least 5000, at least 10,000, at least 50,000, at least 100,000 or more different kinds of the detection reagents. Various kinds of the detection reagents described herein can differ in types of probe reagents (e.g., nucleic acids vs. antibodies), target binding domains, and/or target analytes.

[0063] In some embodiments, the method described herein can further comprise processing the sample before contacting with the composition comprising a plurality of detection reagents described herein. Depending on the types and/or natures of the samples and/or analytes, different sample processing techniques can be used with the methods described herein. Exemplary sample processing techniques include, but are not limited to, mechanical processing of a sample (e.g., without limitations, homogenizing, centrifuging, vortexing, sectioning and shearing), addition of at least one reagent to a sample (e.g., without limitations, lysis buffers, RNA or DNA extraction reagents, RNA or DNA digestion reagents, enzyme inhibitors, fixing agents, organic solvents, antibodies, permeabilizing agents and immunohistochemistry agents), separation of a sample (e.g., without limitations, filtering, centrifuging, electrophoresis, western blot, and Northern blot), mounting a sample on a solid support (e.g., a microscopic slide), and any combinations thereof.

[0064] By way of example only, if a sample is a tissue from a subject (e.g., a biopsy for immunostaining), sample processing can include, but are not limited to, tissue sectioning, mounting on a solid support, fixing the tissue, permeabilizing the tissue (if intracellular proteins are to be detected), blocking non-specific reactions with the detection reagents. In some embodiments, proteins or nucleic acids can be isolated from a tissue or fluid sample and then separated electrophoretically on a separation medium (e.g., electrophoresis gel), followed by transferring the proteins or nucleic acids to a blotting membrane. The blotting membranes containing proteins or nucleic acids can then be contacted with the detection reagents described herein. Methods of processing samples before addition of various types of probe reagents for different kinds of assays are well established in the art, and any of those methods can be performed prior to the contacting step of the methods described herein.

[0065] In some embodiments, the method described herein can further comprise removing any unbound detection reagents before detection of the pre-determined subsequences in a temporally-sequential manner. The term “unbound detection reagents” as used herein refers to detection reagents that have not bound to or interacted with target analytes. The unbound detection reagents can be removed from the sample by any methods known in the art, e.g., rinsing with the sample with a buffered solution at least once, at least two times, at least three times or above.

[0066] After the detection reagents bind to the target analytes in a sample, the nucleic acid labels of the detection reagents carrying nucleic-acid information can be decoded to allow identification of respective probe reagent(s) conjugated to them, as opposed to traditional optical labeling technologies, where an optical signature such as a fluorophore is detected in the absence of providing any nucleic acid information. In embodiments of various aspects described herein, the pre-determined subsequences within the nucleic acid labels are detected in a temporally-sequential manner. The term “temporally-sequential manner” is used in reference to detecting or decoding in a time series a plurality of the pre-determined subsequences within the nucleic acid labels of any detection reagents that are bound to target analytes in a sample. In some embodiments, one or more pre-determined subsequences within at least one nucleic acid label of each detection reagent can be detected or decoded at each time point or detection step of a time series. In some embodiments, one pre-determined subsequence within at least one nucleic acid label of each detection reagent can be detected or decoded at each time point or detection step of

a time series. In some embodiments, at least one pre-determined subsequence (e.g., 1, 2, 3, 4, 5, 6, or more pre-determined subsequences) at the same corresponding location within the nucleic acid label of each detection reagent can be detected or decoded at each time point or detection step of a time series. The time period between any two time points or detection steps can be of any length, e.g., seconds, minutes and hours. For example, the time period between any two time points or detection steps can vary from about 5 seconds to about 2 hours, from about 10 seconds to about 1 hour, from about 30 seconds to about 30 mins, or from about 1 min to about 15 mins. In some embodiments, the time period between any two time points or detection steps can be less than 5 seconds. In other embodiments, the time period between any two time points or detection steps can be longer than 2 hours, longer than 4 hours, longer than 6 hours, longer than 12 hours, longer than 1 day. For example, a sample containing the detection reagents can be maintained at room temperature, at a fridge temperature (e.g., between about 0 °C and about 10°C) or at sub-zero temperatures (e.g., between -80°C or lower and 0°C) during the time period between detection steps. In some embodiments, each subsequent detection step is performed substantially immediately one after another (e.g., within less than 2 seconds, less than 1 second).

[0067] In some embodiments, the pre-determined subsequences can be detected in any temporal orders. In some embodiments, the next pre-determined subsequence to be detected after the previous one can be located closest to the previous one. In some embodiments, the next pre-determined subsequence to be detected after the previous one can be located at least one, at least two, at least three, at least four, at least five or more pre-determined subsequences apart from the previous one. In such embodiments, any pre-determined subsequences that were bypassed in a previous detection step can be detected afterward. In some embodiments of the methods described herein, a computer-implemented software can be used to facilitate an analysis of the temporal readouts from the detection steps, e.g., re-arranging the temporal readouts in an order corresponding to their spatial locations within the nucleic acid label before further comparison and quantification analyses.

[0068] In some embodiments, the detection or decoding of the pre-determined subsequences can comprise nucleic acid sequencing. Methods for sequencing nucleic acids are well established to a skilled artisan, e.g., but not limited to ligation, hybridization, synthesis, amplification or single-base extension, or any combinations thereof. By way of example only, as shown in **Fig. 1** or **Fig. 2**, the nucleic acid labels each contain three pre-determined subsequences (each of one

nucleotide) conjugated together by a direct bond such as a phosphodiester bond. Each sequencing step decodes or determines one nucleotide, wherein each nucleobase (A, G, C or T) generates a distinct signal signature corresponding to the nucleobase. Consequently, a temporal order or time series of the signal signatures generated from each sequencing step corresponds to the respective probe reagent, and thus identify the target analyte. Without wishing to be bound, in this embodiment, the number of sequencing steps performed is not necessarily equal to the number of the pre-determined sequences. In some embodiments, the number of sequencing steps performed can be less than the number of the pre-determined sequences. For example, as shown in **Fig. 1**, two sequencing steps could be sufficient to identify the three different probe reagents, where each base is associated with a different color. However, additional nucleic acid information can increase the accuracy of identifying different probe reagents. Further, if the sequencing of each base can yield one of 4 colors, the n-base subsequences (e.g., 3-base subsequences shown in **Fig. 1** or **Fig. 2**) can produce 4^n possible unique readouts, i.e., 4^n possible distinct probe reagents can be distinguished using such detection reagents described herein.

[0069] While sequencing methods can convey single base difference, other detection or decoding methods that convey information by the presence or absence of entire hybridization “sites” or pre-determined subsequences on the nucleic acid label can also be used for the methods described herein. In some embodiments, the detection step can comprise hybridizing a decoder probe with a subsequence on the nucleic acid label of the detection reagent, wherein the decoder probe can comprise a detectable label. In particular embodiments, the detection method can comprise: (a) hybridizing a set of decoder probes with a subsequence of the detection reagents, wherein each subpopulation of the decoder probes can comprise a detectable label, each detectable label producing a signal signature; (b) detecting said signal signature produced by the hybridization of said set of decoder probes; and (d) repeating steps (a) and (b) for other subsequences of said detection reagents.

[0070] In some embodiments, each subpopulation of the decoder probes can comprise a different detectable label, each different detectable label producing a different signal signature. In these embodiments, the different signal signature produced by the hybridization of the set of decoder probes can be detected.

[0071] In some embodiments, each subpopulation of the decoder probes can be complementary (e.g., partially complementary or completely complementary) to the subsequence

of the detection reagents. In some embodiments, a first subpopulation and a second subpopulation of the decoder probes can be complementary (e.g., partially complementary or completely complementary) to distinct subsequences of the detection reagents. In some embodiments, at least two or more subpopulations of the decoder probes can bind to the same subsequence of the detection reagents. For example, a first subpopulation and a second subpopulation of the decoder probes can be complementary (e.g., partially complementary or completely complementary) to the same subsequence of the detection reagents.

[0072] By way of example only, **Fig. 5** shows an exemplary detection reagent comprising anti-*C. albicans* probe reagents and nucleic acid labels containing three hybridization sites or pre-determined subsequences conjugated together by sequence linkers. In the first hybridization step, a first set of decoder probes each comprising a distinct detectable label (e.g., complementary DNA readout probes shown in **Fig. 5**, each comprising a distinct optical label) is hybridized with a first pre-determined subsequence (e.g., Site 1 in **Fig. 5**), followed by detection of a first signal signature produced by the hybridization. In the second hybridization step, a second set of decoder probes each comprising a distinct detectable label is hybridized with a second pre-determined subsequence (e.g., Site 2 in **Fig. 5**), followed by detection of a second signal signature produced by the hybridization. The second pre-determined subsequence can be the same or different from the first pre-determined subsequence. However, in preferred embodiments, the second pre-determined subsequence is different from the first pre-determined subsequence, e.g., to minimize cross-hybridization with each other. Accordingly, the hybridization and signal detection steps are repeated for other subsequences of the detection reagents with a different set of decoder probes, thereby producing a temporal order or time series of the signal signatures corresponding to the respective probe reagent (and detection reagent).

[0073] As used herein, the term “decoder probe” refers an oligonucleotide with a sequence complementary to a pre-determined sequence of the nucleic acid label. By “complementary” is meant that a nucleic acid can form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non- traditional types. The decoder probe sequence can be completely or partially complementary to a pre-determined sequence. In some embodiments, partial complementarity is indicated by the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9,10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100%

complementary). "completely complementary" or 100% complementarity means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. Less than perfect complementarity refers to the situation in which some, but not all, nucleoside units of two strands can hydrogen bond with each other.

[0074] The decoder probe can have a sequence of any length. In some embodiments, the decoder probe can have a sequence length of about 1 to about 100 nucleotides, about 1 to about 50 nucleotides, about 2 to about 50 nucleotides, about 5 to about 30 nucleotides, or about 5 to about 20 nucleotides.

[0075] In some embodiments, the decoder probe can comprise at least one detectable label described herein. In some embodiments, the detectable label can be an optical label selected from the group consisting of a small-molecule dye, a fluorescent molecule or protein, a quantum dot, a colorimetric reagent, a chromogenic molecule or protein, a Raman label, and any combinations thereof. In some embodiments, the detectable label or optical label can be a fluorescent molecule or protein.

[0076] In some embodiments, the decoder probe can be modified, e.g., base modification or activated with a functional group for linkage to a detectable label.

[0077] The number of decoder probes in each set can vary, depending on the number of distinct subsequences in each hybridization. In some embodiments, there can be about 1 to about 100 decoder probes, about 2 to about 50 decoder probes, about 4 to about 20 decoder probes in each set. In some embodiments, there can be about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50 or more decoder probes in each set. In some embodiments, while each subsequence site can hybridize with a large number of decoder probes, each *set* of decoder probes added in each readout step to hybridize with the subsequence site within the detection reagents can generally have as many as the number of available fluorescent colors. For example, each set of the decoder probes added in each readout step to hybridize with the subsequence site of the detection reagents can contain about 3-4 decoder probes, each of which is labeled with a distinct fluorescent color. In the case of using quantum dots or Raman labels as detection labels, there can be more than 3-4 decoder probes in each set added during each readout step.

[0078] Without wishing to be bound, an example of "non-overlapping nucleic acid labels" or "non-overlapping SeqTag labels" (i.e., no two decoder probes will hybridize with the same

spatial site of the nucleic acid label) is shown herein for illustrative purposes. Assuming there are 12 pre-determined subsequences (e.g., nucleic acid sequences) designed for minimal cross-hybridization with each other and each others' complements (e.g., A1, A2, A3, A4, B1, B2, B3, B4, C1, C2, C3, and C4). Consider a detection reagent comprising a nucleic acid label of the form:



where each position (e.g., A[1-4]) holds only one of the subsequences (e.g., A2). Now, this subsequence is decoded or detected by using decoder probes or complementary probes A1* - A4* each labeled in one of four fluorescent colors. After readout, the signal produced by the fluorophore can be removed (e.g., denaturing to undo the hybridization (and thus removing the fluorophore)) before continuing with B1* - B4*. Since each step yields one of four outcomes, this coding scheme can provide $4 \times 4 \times 4 = 64$ unique readouts. These hybridization-based probes can be used to label 64 different probe reagents that can be read out in three cycles.

[0079] In the case where two decoder probes can overlap, a single site (e.g., A in the above nucleic acid label form), which can accept one of the different decoder probes, can be used. This single site can be read out by subjecting it to the different decoder probes, e.g., in sets of 4 using the nucleic acid label form as shown above. When the correct set is reached, the nucleic acid label, e.g., SeqTag label, should be dark (no-color). This example is not construed to be limiting and any modifications apparent to one of skill in the art is also within the scope of the inventions.

[0080] The advantage of readout by hybridization is that it can be quick: hybridization can take place in minutes or less. Furthermore, no chemistry or enzymes are required during the readout process, as the readout process can be performed by similar methods as used in nucleic acid sequencing, microscopy, spectroscopy, or any combinations thereof. Thus, hybridization-based readout method can reduce cost, reduce reagent storage and/or simplify the process.

[0081] In some embodiments of the methods described herein, there can be no limit in the spatial movement of an analyte in a sample during a temporal detection of the detection reagents, for example, provided that the analyte stay within the field of detection and there is at least one same distinguishable feature in each image taken during a temporal detection so that the images can be aligned to each other based on the same distinguishable feature. In some embodiments where there is no such distinguishable feature, the spatial movement of an analyte in a sample can be less than 100 μm , including less than 50 μm , less than 25 μm , less than 10 μm , less than 1

μm or smaller, over a time period, during which a temporal detection of the detection reagents occurs. In some embodiments, the spatial movement of an analyte in a sample can be less than 1000 nm, including less than 500 nm, less than 250 nm, less than 100 nm, less than 50 nm, less than 10 nm or smaller, over a time period, during which a temporal detection of the detection reagents occurs. More importantly, the spatial movement limit of an analyte in a sample during a temporal detection is determined by the ability of matching distinguishable features between images taken during a temporal detection, which can be affected by imaging conditions. In some embodiments, the analyte can be fixed on a solid substrate or support. In some embodiments where there is or expects to be a spatial movement of an analyte during temporal detection, the location of the analyte with respect to a sample during each detection step can be determined and registered. Such spatial shift can then be corrected afterward during signal analysis using any art-recognized computer-implemented algorithms.

[0082] The length of time required to perform a temporal detection of the detection reagents (e.g., the length of time it takes to obtain a temporal sequence of signal signatures for the detection reagents) can vary, depending on the number of pre-determined subsequences to be detected or read and/or the number of available detection signals (e.g., fluorescent color and/or brightfield) to be read. In some embodiments, the length of time required to perform a temporal detection of the detection reagents can be, for example, but not limited to, 5 seconds, 10 seconds, 15 seconds, 20 seconds, 30 seconds, 1 mins, 2 mins, 3 mins, 4 mins, 5 mins, 15 mins, 30 mins, 1 hour, 2 hours, 4 hours, 6 hours, or longer.

[0083] The detection reagents can be detected by any means available in the art that is capable of detecting the specific signals on a given detection reagent generated during sequencing- or hybridization-based methods. Where the detection reagents (e.g., hybridized with decoder probes) are fluorescently labeled, suitable consideration of appropriate excitation sources can be readily determined. Possible sources can include but are not limited to arc lamp, xenon lamp, lasers, light emitting diodes or some combination thereof. The appropriate excitation source is used in conjunction with an appropriate optical detection system, for example an inverted fluorescent microscope, an epi-fluorescent microscope or a confocal microscope. Preferably, a microscope is used that can allow for detection with enough spatial resolution to separate distinct signals from individual detection reagents.

[0084] Exemplary methods for detection of the detection reagents that are applicable to the methods described herein include, without limitations, the methods described in U.S. Pat. No. 7,473,767, US patent publication no. 2007/0166708, and US application number US 2010/0261026, all of which are incorporated by reference herein in its entirety.

[0085] Additional methods that can be used to detect optical signatures include, but are not limited to, any spectroscopic techniques, flow cytometry, or any art-recognized methods involving an optical scanner and/or a photodetector (e.g., without limitations, a charge-coupled devices, active pixel sensors, photodiode light sensors (e.g., LEDs), optical detectors, and any combinations thereof). Non-limiting examples of spectroscopic techniques can include absorption spectroscopy, emission spectroscopy, elastic scattering spectroscopy, reflection spectroscopy, impedance spectroscopy, inelastic spectroscopy, coherent or resonance spectroscopy, surface plasmon fluorescence spectroscopy, Raman spectroscopy, and any combinations thereof. Spectroscopy techniques can be used to detect light of any wavelengths, including, but not limited to, microwave, terahertz, infrared, near infrared, visible, ultraviolet, x-ray, gamma, and any combinations thereof.

[0086] Without wishing to be limited, in some embodiments, at least one pre-determined subsequence (e.g., individual bases or hybridization regions) can correspond to no optical signature; that is the absence of color can be considered as an additional color. In other embodiments, at least one pre-determined subsequence (e.g., individual bases or hybridization regions) can correspond to a compound optical signature, e.g., two or more simultaneous fluorescence in multiple channels during detection (e.g., by microscopy).

[0087] While a single area of a sample can interact with more than one probe reagents, the nucleic acid label can be designed such that any known or potential overlaps could be teased apart from the signal output. In the case where any probe may potentially overlap with all others, one can use the readout-by-hybridization variation and assign each probe a single unique hybridization sequence. Such approach can avoid multiple lengthy probe incubations and damaging stripping steps.

[0088] In some embodiments of the methods described herein, the signal signatures produced during any readout step (e.g., sequencing-based or hybridization-based) should be removed before advancing to the next pre-determined subsequence of the detection reagents. The removal of the signal signatures can be done by any methods known in the art, including, but not limited

to, washing, heating, photo-bleaching, displacement, cleavage, enzymatic digestion, quenching, chemical degradation, bleaching, oxidation, and any combinations thereof.

[0089] In some embodiments, the decoder probes can be designed such that they can be simply washed out either with a plain buffer, or they can be modified by varying salt concentrations or using detergents or denaturants such as formamide or dimethyl sulfoxide (DMSO).

[0090] In other embodiments, the fluorescence or color signature of a readout step can be attenuated or eliminated by photo-bleaching the signal using sufficient optical exposure. In alternative embodiments, the fluorescence or color signature of a readout step can be attenuated or eliminated by subjecting the fluorescence or color signature to chemical degradation under appropriate conditions, e.g., using a reducing agent or oxidizing solution such as 0.01 M sodium periodate.

[0091] In some embodiments, the decoder probes can be displaced from their hybridization sites by introducing other reagents or probe displacers that have stronger binding affinities to those same sites. This can be done, for example, by using nucleic acid sequences that are longer than and/or have better complementarity than the decoder probe sequences. For example, to create “better complementarity” of the probe displacers, in some embodiments, mismatches can be seeded in the hybridization region. In other embodiments, the hybridization region can be preceded and/or post-ceded with a “toe-hold” of around 3-8 bases (e.g., 6 bases). In such embodiments, the “better complementarity” of the probe displacer can be outside of the hybridization region, which can make the design of the hybridization regions easier.

[0092] In some embodiments, enzymes can be used to displace, digest, cut and/or cleave the detectable labels, the decoder probe sequence, the hybridized complex (formed by the decoder probe and pre-determined subsequence), and/or the cleavable sequence linker to the hybridized complex, in order to remove the signal signatures. One example is to introduce a deoxyuridine into the decoder probe. This modified base can be cleaved using the enzyme mix known as USER, thereby cutting the decoder probe sequence into two parts. Since each part is now shorter, it is characterized by a lower melting temperature and can melt off the hybridization sites of the detection reagents. Alternatively, one of skill in the art can employ one of numerous art-recognized sequence-specific nucleases or restriction enzymes, which can cut either the decoder probe sequence, the pre-determined subsequence that has been hybridized with decoder probes,

the cleavable sequence linker attached to the hybridized subsequence and/or the hybridized complex thereof, thereby removing the signal signature.

[0093] In some embodiments, thermal denaturing can be used to remove the signal signature from a previous readout. In some embodiments where sequencing is involved, thermal denaturing can be reduced or avoided by using a sequencing-by-ligation approach and by setting the nucleic acid label base that immediately follows the sequencing primer (in the direction of ligation) to an adenine. Correspondingly, the ligation probes should then include a (deoxy-)uracil in the primer-proximal position. Without wishing to be bound by theory, an enzyme such as USER, which cleaves DNA at uracils can be used to remove the ligation probe and ready the system for the next sequencing step. These fragments will have lower melting temperatures than their parent probes, and these temperatures can be designed to fall below the operating temperature (or require an acceptable denaturing temperature).

[0094] After detection of the pre-determined subsequences is completed in a temporally-sequential manner, in some embodiments, the method described herein can further comprise comparing the temporal order of the signal signatures with different identifiers of said at least one probe reagent, wherein an agreement between the temporal order of the signal signatures and a particular identifier of said at least one probe reagent identifies the analyte in the sample. In some embodiments, the method can further comprise measuring the intensity of the signal signatures generated from each subpopulation of the detection reagents. In some embodiments, the intensity of the signal signatures generated from each subpopulation of the detection reagents can indicate an amount of the analyte. In some embodiments, the relative intensity of the signal signatures can be used in identification of each subpopulation of the detection reagents. Thus, the intensity of the signal signatures can be used as part of a coding scheme of the detection reagents described herein. The comparing and intensity measuring steps can be performed, e.g., by a computer-implemented software or algorithm.

[0095] Types of signal signature(s) can vary upon different embodiments of detection reagents and/or decoder probes described herein. As used herein, the term “signal signature” refers to a change in, or occurrence of, a response or indicator that is detectable either by observation or instrumentally. In certain instances, the signal signature is fluorescence or a change in fluorescence, e.g., a change in fluorescence intensity, fluorescence excitation or emission wavelength distribution, fluorescence lifetime, and/or fluorescence polarization. By

way of example only, the fluorescence can be produced by binding a fluorophore to a decoder probe, and/or by detecting the hybridization using a fluorescent dye, e.g., SYBR Gold, that lights up when nucleic acid sequence becomes double-stranded. In certain other instances, the signal signature can be radioactivity (i.e., radiation), including alpha particles, beta particles, nucleons, electrons, positrons, neutrinos, and gamma rays emitted by a radioactive substance such as a radionuclide. By way of example, the detection reagents and/or decoder probes can comprise an optical molecule or label, thus producing optical signatures. Examples of optical signatures can include, without limitations, signatures of fluorescent color, visible light, no-color, and any combinations thereof. In such embodiments, the optical signatures can be detected by optical imaging or spectroscopy.

Detection reagents (or detection molecules as used interchangeably herein)

[0096] Another aspect provided herein is a detection reagent, which can be, for example, used in the methods described herein for any multiplexing assays. The detection reagent comprises at least one probe reagent and at least one nucleic acid label, wherein said at least one nucleic acid label comprises at least one pre-determined subsequence to be detected in a temporally-sequential manner; wherein said at least one pre-determined subsequence forms an identifier of said at least one probe reagent; and wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together.

[0097] The detection reagents described herein can exist in different forms. By way of example only, in some embodiments, the detection reagent can be a detection molecule. In some embodiments, the detection reagent can be a detection particle. In some embodiments, the detection reagent can be multi-molecular.

[0098] As used herein, the term "conjugated" refers to two molecules being linked to each other, e.g., attaching a probe reagent to a nucleic acid label. The conjugation process can be performed, e.g., via a chemical reaction, or via a linker, which will be described later.

[0099] Depending on various applications and/or assay conditions (e.g., sensitivity, sample volume/concentration), a readout signal of a detection reagent can be amplified by increasing the number of the nucleic acid labels present in the detection reagent, e.g., by conjugating at least one probe reagent to a plurality of nucleic acid labels. In such embodiments, a plurality of the nucleic acid labels present in the detection reagent can range from about 2 to about 100,000,

about 2 to about 10,000, about 2 to about 1,000, or about 2 to about 100. In some embodiments where the detection reagent comprises a particle as a hub, the number of possible nucleic acid labels present in the detection reagent can depend on the size of a particle. Generally, the larger the particle it is, the more nucleic acid labels can be incorporated into the detection reagent. For example, a particle of about 1-2 μm in size can allow incorporation of about 100,000 nucleic acid labels into the detection reagent. In some embodiments, there can be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 5000, 10000, 50000, 100000 nucleic acid labels present in the detection reagent. One of skill in the art can determine the optimum number of nucleic acid labels present the detection reagent without any undue experimentation.

[00100] The detection reagents described herein can be used in any biological assays for detection, identification and/or quantification of target molecules or analytes, including counting marked cells such as bacteria or cancer cells, in a sample. By way of example only, in some embodiments, the detection reagent can be adapted for use in immunofluorescence. In alternative embodiments, the detection reagent can be adapted for use in immunohistochemistry. In other embodiments, the detection reagent can be adapted for use in fluorescence in situ hybridization. In some embodiments, the detection reagent can be adapted for use in western blot. Depending on the nature of the sample and/or applications, the detection reagent can be adapted to be in any format, e.g., immobilized on a solid support, or in a solution or suspension phase. In certain embodiments, the detection reagent can be adapted to be present in a solution or suspension phase. The phrase “in a solution or suspension phase” as used herein generally refers to suspending the detection reagents in a liquid fluid, e.g., an aqueous buffer solution. Additional applications of the detection reagents and/or methods described herein will be discussed.

Probe reagents (or probe molecules as used interchangeably herein)

[00101] Each of the detection reagents described herein can comprise any number of probe reagents. In some embodiments, the detection reagent can comprise one or more probe reagents, e.g., at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10 or more probe reagents. In one embodiment, the detection reagent can comprise one probe reagent. In other embodiments, the detection reagent can comprise a plurality of probe reagents, e.g., ranging from about 2 to about 100,000 probe reagents, about 2 to about 10,000 probe reagents, about 2 to about 1,000 probe reagents, or about 2 to about 100 probe reagents. In

some embodiments where the detection reagent comprises a particle as a hub, the number of possible probe reagents present in the detection reagent can depend on the size of a particle. Generally, the larger the particle it is, the more probe reagents can be incorporated into the detection reagent. For example, a particle of about 1-2 μm in size can allow incorporation of about 100,000 probe reagents into the detection reagent. In some embodiments, there can be about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 5000, 10000, 50000, 100000 probe reagents present in the detection reagent. One of skill in the art can determine the optimum number of probe reagents present the detection reagent without any undue experimentation.

[00102] As used interchangeably herein, the term "probe," "probe reagent" or "probe molecule" refers to an entity (e.g., but not limited to, a molecule, a particle, a composite entity, or a multi-molecular entity) that interacts with or binds to a target molecule or an analyte for the analysis of the target or the analyte. Typically the nature of the interaction or binding is noncovalent, e.g., by hydrogen, electrostatic, or van der Waals interactions, however, binding can also be covalent. Probe reagents can be entities (e.g., but not limited to, molecules, a particles, composite entities, or multi-molecular entities) capable of undergoing binding or molecular recognition events with target molecules. Probe reagents can be naturally-occurring, recombinant or synthetic. Examples of the probe reagent can include, but are not limited to, a nucleic acid, an antibody or a portion thereof, an antibody-like molecule, an enzyme, a cell, an antigen, a small molecule, a protein, a peptide, a peptidomimetic, an aptamer, and any combinations thereof. By way of example only, in immunohistochemistry, the probe reagent can include an antibody specific to the target antigen to be analyzed. An ordinary artisan can readily identify appropriate probe reagents for the target molecules or analytes of interest to be detected in various bioassays. In some embodiments, the probe reagent can be multi-molecular. For example, in one embodiment, the probe reagent can comprise a particle, an antibody, biotin and/or streptavidin, or any combinations thereof.

[00103] In some embodiments, the probe reagents can be modified by any means known to one of ordinary skill in the art. Methods to modify each type of probe reagents are well recognized in the art. Depending on the types of probe reagents, an exemplary modification includes, but is not limited to genetic modification, biotinylation, labeling (for detection purposes), chemical modification (e.g., to produce derivatives or fragments of the probe reagent),

and any combinations thereof. In some embodiments, the probe reagent can be genetically modified. In some embodiments, the probe reagent can be biotinylated.

[00104] As used herein, the terms "proteins" and "peptides" are used interchangeably herein to designate a series of amino acid residues connected to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. The terms "protein", and "peptide", which are used interchangeably herein, refer to a polymer of protein amino acids, including modified amino acids (e.g., phosphorylated, glycosylated, etc.) and amino acid analogs, regardless of its size or function. Although "protein" is often used in reference to relatively large polypeptides, and "peptide" is often used in reference to small polypeptides, usage of these terms in the art overlaps and varies. The term "peptide" as used herein refers to peptides, polypeptides, proteins and fragments of proteins, unless otherwise noted. The terms "protein" and "peptide" are used interchangeably herein when referring to a gene product and fragments thereof. Thus, exemplary peptides or proteins include gene products, naturally occurring proteins, homologs, orthologs, paralogs, fragments and other equivalents, variants, fragments, and analogs of the foregoing.

[00105] As used herein, the term "peptidomimetic" refers to a molecule capable of folding into a defined three-dimensional structure similar to a natural peptide

[00106] The term "nucleic acids" used herein refers to polymers (polynucleotides) or oligomers (oligonucleotides) of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars and intersugar linkages. The term "nucleic acid" also includes polymers or oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly. Exemplary nucleic acids include, but are not limited to, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), locked nucleic acid (LNA), peptide nucleic acids (PNA), and polymers thereof in either single- or double-stranded form. Locked nucleic acid (LNA), often referred to as inaccessible RNA, is a modified RNA nucleotide. The ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' oxygen and 4' carbon. The bridge "locks" the ribose in the 3'-endo conformation. LNA nucleotides can be mixed with DNA or RNA residues in the oligonucleotide whenever desired. Such LNA oligomers are generally synthesized chemically. Peptide nucleic acid (PNA) is an artificially synthesized polymer similar to DNA or RNA. DNA and RNA have a deoxyribose and ribose sugar backbone, respectively, whereas PNA's backbone is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. PNA is generally synthesized chemically. Unless specifically limited, the term "nucleic

acids" encompasses nucleic acids containing known analogs of natural nucleotides, which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzner, et al., *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka, et al., *J. Biol. Chem.* 260:2605-2608 (1985), and Rossolini, et al., *Mol. Cell. Probes* 8:91-98 (1994)). The term "nucleic acid" should also be understood to include, as equivalents, derivatives, variants and analogs of either RNA or DNA made from nucleotide analogs, and, single (sense or antisense) and double-stranded polynucleotides.

[00107] In some embodiments, the term "nucleic acid" described herein can include a modified nucleic acid. Modified nucleic acids are well known in the art. Thus, a nucleic acid described herein can comprise one or more nucleic acid modifications known in the art. For example, the nucleic acid can comprise one or more nucleic acid modifications selected from the group consisting of internucleotide linkage modifications (intersugar linkage modifications), sugar modifications, nucleobase modifications, backbone modifications/replacements, and any combinations thereof. Exemplary internucleotide linkage modifications include, but are not limited to, phosphorothioate, phosphorodithioate, phosphotriester (e.g. alkyl phosphotriester), aminoalkylphosphotriester, alkyl-phosphonate (e.g., methyl-phosphonate), selenophosphate, phosphoramidate (e.g., N-alkylphosphoramidate), boranophosphonate, and the like. Exemplary sugar modifications include, but are not limited to, 2'-O-Me (2'-O-methyl), 2'-O-MOE (2'-O-methoxyethyl), 2'-F, 2'-O-[2-(methylamino)-2-oxoethyl] (2'-O-NMA), 2'-S-methyl, 2'-O-CH₂-(4'-C) (LNA), 2'-O-CH₂CH₂-(4'-C) (ENA), 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethoxyethyl (2'-O-DMAEOE), arabinose sugar, and the like. Exemplary nucleobase modifications include, but are not limited to, inosine, xanthine, hypoxanthine, nubularine, isoguanisine, tubercidine, 5-methylcytosine (5-me-C); 5-hydroxymethyl cytosine; xanthine; hypoxanthine; 2-aminoadenine; 6-methyl and other 6-alkyl derivatives of adenine and guanine; 2-propyl and other 2-alkyl derivatives of adenine and guanine; 2-thiouracil; 2-

thiothymine; 2-thiocytosine; 5-propynyl uracil; 5-propynyl cytosine; 6-azouracil; 6-azocytosine; 6-azothymine; 5-uracil (pseudouracil); 4-thiouracil; 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines; 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines; 7-methyl and other 7-alkyl derivatives of adenine and guanine; 8-azaguanine; 8-azaadenine; 7-deazaguanine; 7-deazaadenine; 3-deazaguanine; 3-deazaadenine; universal base; and any combinations thereof. Exemplary backbone modifications include, but are not limited to, morpholino, cyclobutyl, pyrrolidine, peptide nucleic acid (PNA), aminoethylglycyl PNA (*aeg*PNA), backbone-extended pyrrolidine PNA (*bep*PNA), and the like.

[00108] The term “enzymes” as used here refers to a protein molecule that catalyzes chemical reactions of other substances without it being destroyed or substantially altered upon completion of the reactions. The term can include naturally occurring enzymes and bioengineered enzymes or mixtures thereof. Examples of enzyme families include kinases, dehydrogenases, oxidoreductases, GTPases, carboxyl transferases, acyl transferases, decarboxylases, transaminases, racemases, methyl transferases, formyl transferases, and α -ketodecarboxylases.

[00109] As used herein, the term “aptamers” means a single-stranded, partially single-stranded, partially double-stranded or double-stranded nucleotide sequence capable of specifically recognizing a selected non-oligonucleotide molecule or group of molecules. In some embodiments, the aptamer recognizes the non-oligonucleotide molecule or group of molecules by a mechanism other than Watson-Crick base pairing or triplex formation. Aptamers can include, without limitation, defined sequence segments and sequences comprising nucleotides, ribonucleotides, deoxyribonucleotides, nucleotide analogs, modified nucleotides and nucleotides comprising backbone modifications, branchpoints and nonnucleotide residues, groups or bridges. Methods for selecting aptamers for binding to a molecule are widely known in the art and easily accessible to one of ordinary skill in the art.

[00110] As used herein, the term “antibody” or “antibodies” refers to an intact immunoglobulin or to a monoclonal or polyclonal antigen-binding fragment with the Fc (crystallizable fragment) region or FcRn binding fragment of the Fc region. The term “antibodies” also includes “antibody-like molecules”, such as fragments of the antibodies, e.g., antigen-binding fragments. Antigen-binding fragments can be produced by recombinant DNA techniques or by enzymatic or chemical cleavage of intact antibodies. “Antigen-binding

fragments" include, inter alia, Fab, Fab', F(ab')₂, Fv, dAb, and complementarity determining region (CDR) fragments, single-chain antibodies (scFv), single domain antibodies, chimeric antibodies, diabodies, and polypeptides that contain at least a portion of an immunoglobulin that is sufficient to confer specific antigen binding to the polypeptide. Linear antibodies are also included for the purposes described herein. The terms Fab, Fc, pFc', F(ab')₂ and Fv are employed with standard immunological meanings (Klein, Immunology (John Wiley, New York, N.Y., 1982); Clark, W. R. (1986) The Experimental Foundations of Modern Immunology (Wiley & Sons, Inc., New York); and Roitt, I. (1991) Essential Immunology, 7th Ed., (Blackwell Scientific Publications, Oxford)). Antibodies or antigen-binding fragments specific for various antigens are available commercially from vendors such as R&D Systems, BD Biosciences, e-Biosciences and Miltenyi, or can be raised against these cell-surface markers by methods known to those skilled in the art.

[00111] As used herein, the term "Complementarity Determining Regions" (CDRs; i.e., CDR1, CDR2, and CDR3) refers to the amino acid residues of an antibody variable domain the presence of which are necessary for antigen binding. Each variable domain typically has three CDR regions identified as CDR1, CDR2 and CDR3. Each complementarity determining region may comprise amino acid residues from a "complementarity determining region" as defined by Kabat (i.e. about residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (i.e. about residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk J. Mol. Biol. 196:901-917 (1987)). In some instances, a complementarity determining region can include amino acids from both a CDR region defined according to Kabat and a hypervariable loop.

[00112] The expression "linear antibodies" refers to the antibodies described in Zapata et al., Protein Eng., 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (VH-CH1-VH-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

[00113] The expression "single-chain Fv" or "scFv" antibody fragments, as used herein, is intended to mean antibody fragments that comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. (Plückthun, *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer- Verlag, New York, pp. 269-315 (1994)).

[00114] The term "diabodies," as used herein, refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) Connected to a light-chain variable domain (VL) in the same polypeptide chain (VH - VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. (EP 404,097; WO 93/11161; Hollinger et al, *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993)).

[00115] As used herein, the term "small molecules" refers to natural or synthetic molecules including, but not limited to, peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, aptamers, nucleotides, nucleotide analogs, organic or inorganic compounds (i.e., including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

[00116] The term "cells" used herein refers to any cell, prokaryotic or eukaryotic, including plant, yeast, worm, insect and mammalian. Mammalian cells include, without limitation; primate, human and a cell from any animal of interest, including without limitation; mouse, hamster, rabbit, dog, cat, domestic animals, such as equine, bovine, murine, ovine, canine, feline, etc. The cells may be a wide variety of tissue types without limitation such as; hematopoietic, neural, mesenchymal, cutaneous, mucosal, stromal, muscle spleen, reticuloendothelial, epithelial, endothelial, hepatic, kidney, gastrointestinal, pulmonary, T-cells etc. Stem cells, embryonic stem (ES) cells, ES- derived cells and stem cell progenitors are also included, including without

limitation, hematopoietic, neural, stromal, muscle, cardiovascular, hepatic, pulmonary, gastrointestinal stem cells, etc. Yeast cells may also be used as cells in some embodiments of the methods described herein. In some embodiments, the cells can be ex vivo or cultured cells, e.g. in vitro. For example, for ex vivo cells, cells can be obtained from a subject, where the subject is healthy and/or affected with a disease. Cells can be obtained, as a non-limiting example, by biopsy or other surgical means known to those skilled in the art.

[00117] As used herein, the term "antigens" refers to a molecule or a portion of a molecule capable of being bound by a selective binding agent, such as an antibody, and additionally capable of being used in an animal to elicit the production of antibodies capable of binding to an epitope of that antigen. An antigen may have one or more epitopes. The term "antigen" can also refer to a molecule capable of being bound by an antibody or a T cell receptor (TCR) if presented by MHC molecules. The term "antigen", as used herein, also encompasses T-cell epitopes. An antigen is additionally capable of being recognized by the immune system and/or being capable of inducing a humoral immune response and/or cellular immune response leading to the activation of B- and/or T-lymphocytes. This may, however, require that, at least in certain cases, the antigen contains or is linked to a Th cell epitope and is given in adjuvant. An antigen can have one or more epitopes (B- and T-epitopes). The specific reaction referred to above is meant to indicate that the antigen will preferably react, typically in a highly selective manner, with its corresponding antibody or TCR and not with the multitude of other antibodies or TCRs which may be evoked by other antigens. Antigens as used herein may also be mixtures of several individual antigens.

[00118] In some embodiments, the probe reagent can be an antibody or a portion thereof, or an antibody-like molecule. In such embodiments, the probe reagents can be used to, for example, detect and/or identify pathogen type or species, the presence of cell or disease markers, cellular protein expression levels, phosphorylation or other post-translation modification state, or any combinations thereof. By way of example only, **Fig. 1** shows three different embodiments of the detection reagents comprising at least one (e.g., 1, 2, 3, 4, 5 or more) pathogen-specific antibodies (e.g., anti-E. Coli, anti-S. aureus, and anti-C. albicans).

[00119] In some embodiments, the probe reagent can be a nucleic acid (e.g., DNA, RNA, LNA, PNA, or any combinations thereof). In such embodiments, the nucleic acids can be used to determine, for example, the existence of characteristic cellular DNA or RNA sequences (such as

in fluorescent in situ hybridization), RNA expression levels, miRNA presence and expression, and any combinations thereof, in various applications, e.g., for pathogen detection and/or identification.

[00120] In some embodiments, the probe reagent can be a protein or a peptide. In such embodiments, the protein or peptide can be essentially any proteins with known binding targets. Examples include, but are not limited to, innate-immune proteins (e.g., without limitations, MBL, Dectin-1, TLR2, and TLR4 and any proteins disclosed in U.S. Provisional Application No.: 61/508,957, the content of which is incorporated herein by reference in its entirety) and proteins comprising the chitin-binding domain. Such innate-immune proteins and chitin-binding domain proteins can be used to detect their corresponding pattern-recognition targets (e.g., microbes such as bacteria) and fungus, respectively. By way of example only, instead of using pathogen-specific antibodies as probe reagents in the detection reagents as shown in **Fig. 1**, innate-immune proteins (e.g., MBL) or chitin-binding domain proteins can be used as probe reagents for detection of pathogens. While such detection reagents can be used to detect pathogens, they may not be pathogen-specific, as compared to the ones using pathogen-specific antibodies as probes molecules.

[00121] In some embodiments, the probe reagent can be an aptamer. In some embodiments, the probe reagent can be a DNA or RNA aptamer. The aptamers can be used in various bioassays, e.g., in the same way as antibodies or nucleic acids described herein. By way of example only, **Fig. 2** shows some exemplary embodiments of the detection reagents comprising at least one (e.g., 1, 2, 3, 4, 5 or more) DNA aptamers (e.g., with a nucleotide sequence complementary to nuclear reprogramming factors, such as Oct4, Sox2, and Klf4). Such detection reagents can be used to determine RNA expression level of nuclear reprogramming factors in somatic cells for detecting, screening, or identifying stem cells (e.g., induced pluripotency stem cells).

[00122] In some embodiments, the probe reagent can be a cell surface receptor ligand. As used herein, a “cell surface receptor ligand” refers to a molecule that can bind to the outer surface of a cell. Exemplary, cell surface receptor ligand includes, for example, a cell surface receptor binding peptide, a cell surface receptor binding glycopeptide, a cell surface receptor binding protein, a cell surface receptor binding glycoprotein, a cell surface receptor binding organic compound, and a cell surface receptor binding drug. Additional cell surface receptor

ligands include, but are not limited to, cytokines, growth factors, hormones, antibodies, and angiogenic factors.

[00123] When the detection reagents described herein are used as targeted delivery vehicles, e.g., for a diagnostic agent, in some embodiments, the probe reagent can be an endosomolytic ligand. As used herein, the term “endosomolytic ligand” refers to molecules having endosomolytic properties. Endosomolytic ligands can promote the lysis of and/or transport of the composition described herein, or its components, from the cellular compartments such as the endosome, lysosome, endoplasmic reticulum (ER), Golgi apparatus, microtubule, peroxisome, or other vesicular bodies within the cell, to the cytoplasm of the cell. Some exemplary endosomolytic ligands include, but are not limited to, imidazoles, poly or oligoimidazoles, linear or branched polyethyleneimines (PEIs), linear and branched polyamines, e.g. spermine, cationic linear and branched polyamines, polycarboxylates, polycations, masked oligo or poly cations or anions, acetals, polyacetals, ketals/polyketals, orthoesters, linear or branched polymers with masked or unmasked cationic or anionic charges, dendrimers with masked or unmasked cationic or anionic charges, polyanionic peptides, polyanionic peptidomimetics, pH-sensitive peptides, natural and synthetic fusogenic lipids, natural and synthetic cationic lipids.

[00124] In other embodiments, the probe reagent for use in delivery of an agent (e.g., a diagnostic agent) encapsulated within the detection reagents described herein can be a PK modulating ligand. As used herein, the terms “PK modulating ligand” and “PK modulator” refers to molecules which can modulate the pharmacokinetics of the composition described herein. Some exemplary PK modulator include, but are not limited to, lipophilic molecules, bile acids, sterols, phospholipid analogues, peptides, protein binding agents, vitamins, fatty acids, phenoxazine, aspirin, naproxen, ibuprofen, suprofen, ketoprofen, (S)-(+)-pranoprofen, carprofen, PEGs, biotin, and transthyretin-binding ligands (e.g., tetraiodothyroacetic acid, 2, 4, 6-triiodophenol and flufenamic acid).

[00125] In various embodiments, the detection reagent described herein can comprise one kind/species of probe reagents or different kinds/species of probe reagents. In some embodiments, the kind/species of the probe reagents present in the detection reagent can be the same. In other embodiments, the detection reagent can include at least one different kind/species of the probe reagents (e.g., 1, 2, 3, 4, 5, or 6 probe reagent species). In such embodiments, the

distinct probe reagent species can be different from the others by types (e.g., antibodies vs. DNA aptamers), binding domains, and/or target analytes.

Nucleic acid labels

[00126] In accordance with embodiments of various aspects described herein, the nucleic acid label or nucleic acid tag comprises at least one pre-determined nucleic acid subsequence, which is used to identify an analyte or target. In some embodiments, the nucleic acid label or nucleic acid tag can comprise any number of the pre-determined nucleic acid subsequences, e.g., ranging from about 1 to about 100, from about 2 to about 80, or from about 3 to about 50. In some embodiments, the nucleic acid label or nucleic acid tag can comprise at least about 1, at least about 2, at least about 3, at least about 4, at least about 5, at least about 6, at least about 7, at least about 8, at least about 9, at least about 10, at least about 15, at least about 20, at least about 30, at least about 40, at least about 50, at least about 60, at least about 70 or more pre-determined nucleic acid subsequences. In some embodiments, the nucleic acid label or nucleic acid tag can comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70 or more pre-determined nucleic acid subsequences. Without wishing to be bound, the minimum number of pre-determined nucleic acid subsequences (n) required in the detection reagent can vary upon the number of distinct probes to be detected (X) and/or the number of distinct detectable labels (e.g., optical labels such as fluorescent labels or quantum dots) available to be used (Y), and n can be determined by the equation: $n = \text{ceiling}(\frac{\ln X}{\ln Y})$, where the mathematical function “ceiling” refers to rounding up a non-integer number to the nearest integer, when needed. For example, if 4 distinct detectable labels (Y) are used to distinguish 62 distinct probe reagents (X), $n = \text{ceiling}(2.98) \sim 3$. Therefore, at least three pre-determined nucleic acid subsequences are required in this example.

[00127] In some embodiments where the detectable labels include no-color (dark), the number of distinct detectable label available to be used can become $Y+1$. In such embodiments, n can be determined by the equation: $n = \text{ceiling}(\frac{\ln X}{\ln(Y+1)})$, and thus fewer pre-determined nucleic acid subsequences can be used. However, the combination in which all readout cycles are dark should not be allowed.

[00128] Further, the pre-determined nucleic acid subsequences can be designed such that each readout cycle can light up in multiple colors. Thus, 2^Y instead of Y is used in the equation for n above and fewer pre-determined nucleic acid subsequences can thus be required. However, the combination in which all readout cycles are dark should not be allowed.

[00129] Additionally, while colors are generally used in a binary fashion, i.e., whether the color is present or not, in the above examples, the color intensity can also be used as a parameter of a signal signature. For example, if one detectable label is allowed to light up twice as brightly in a different color than another, the multiplexing capacity can be even further expanded.

[00130] The pre-determined nucleic acid subsequences can be constructed from any types of nucleic acids, including, but not limited to, DNA, RNA, PNA, LNA and any combinations thereof.

[00131] Each of the pre-determined subsequences of the nucleic acid label can be independently of any length. In certain embodiments, the pre-determined subsequences can each independently comprise a length of about 1 nucleobase to about 100 nucleobases, from about 1 nucleobase to about 50 nucleobases, from about 2 nucleobases to about 50 nucleobases, from about 5 nucleobases to about 30 nucleobases, or from about 5 nucleobases to about 20 nucleobases. In some embodiments, the pre-determined subsequences can each independently comprise one or more nucleobases, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more nucleobases.

[00132] The achievable length of the pre-determined subsequences can affect the degree of multiplexability. In some embodiments, the achievable length of the pre-determined subsequences can be a function of the decreasing fluorescence intensity over many cycles of stripped and rehybridization (i.e. diffusion of the sequenceable particles, degradation, increasing background noise). In the case of DNA or RNA based nucleic acid labels that are amplified in situ, modified base is incorporated during its synthesis (i.e., dUTP, biotin, Acrydite, aminoallele), enabling these detection reagents to be permanently embedded in a film (regardless of the film thickness) of functionalized polyacrylamide (i.e. Acrydite streptavidin, NHS ester Acrydite). The embedded material can then be stripped and rehybridized for many more cycles without altering its spatial architecture and minimizing the background noise.

[00133] Two or more pre-determined subsequences can be conjugated together within a nucleic acid label using any methods known in the art. In some embodiments, two or more pre-

determined subsequences can be conjugated together by a sequence linker. The term “sequence linker” as used herein generally refers to an entity that connects two sequences or subsequences as described herein together.

[00134] In some embodiments, the sequence linker can be a direct bond or an atom such as nitrogen, oxygen or sulfur; a unit such as NR_1 , $\text{C}(\text{O})$, $\text{C}(\text{O})\text{NH}$, SO , SO_2 , SO_2NH ; or a chain of atoms. If needed, the two ends of the pre-determined subsequences can be linked together by providing on the two ends of the pre-determined subsequences complementary chemical functionalities that undergo a coupling reaction. In particular embodiments, the sequence linker is a direct bond, including, but not limited to, a phosphodiester bond. For example, a 3' carbon atom of a sugar base at the 5' end nucleotide of a first pre-determined subsequence can interact with the 5' carbon atom of another sugar base at the 3' end nucleotide of a second pre-determined subsequence to form a covalent bond, e.g., a phosphodiester bond. As such, two or more pre-determined subsequences can be bonded together to form a longer and contiguous pre-determined subsequence.

[00135] In some embodiments, the sequence linker can be a nucleotidic linker. The term “nucleotidic linker” as used herein refers to a linker of one nucleotide long or a sequence substantially comprising a plurality of nucleotides. In some embodiments, the nucleotidic linker can have a sequence length of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 30 or more nucleotides. The sequence length of the nucleotidic linker can vary with a number of factors, e.g., detection methods, and/or properties of optical labels. Without wishing to be bound by theory, in some embodiments, increasing the length of the nucleotidic linker can increase the flexibility of the nucleic acid label, e.g., to increase the binding frequency between a pre-determined sequence and a decoder probe, the term of which will be discussed later. However, too long a nucleotidic linker can result in a too long nucleic acid label, which could overlap with other nucleic acid labels of the detection reagents during an assay and thus reduce the quality and/or accuracy of the signal detection. One of skill in the art can determine the optimum length of the nucleotidic linker without undue experimentations.

[00136] The nucleotidic linker can be in any structure or conformation. In some embodiments, the nucleotidic linker can be in a structure selected from the group consisting of single-stranded, double-stranded, partially double-stranded, a hairpin, or any combinations thereof.

[00137] In some embodiments, the sequence linker can be a bead or a nanoparticle acting as a hub. Accordingly, two or more pre-determined subsequences can be independently conjugated together via a bead or a nanoparticle.

[00138] Without wishing to be bound, while the sequence linker can be a direct bond, an atom, a nucleotidic linker, or any combinations thereof, the sequence linker can also include a sequence of amino acids, a polymer chain, a microbead, a nanobead, or any combinations thereof. To provide for the linkages between sequence linker, in some embodiments, different functionalities can be introduced to the ends of sequence linker and/or the pre-determined subsequences. Examples of functionalities include, but are not limited to, amide groups, including carbonic acid derivatives, ethers, esters, including organic and inorganic esters, amino, urethane, urea and any combinations thereof.

[00139] In some embodiments, the nucleic acid label is substantially a polynucleotide sequence containing one or more pre-determined subsequences. In such embodiments, any two pre-determined subsequences are either joined or conjugated together by a direct bond such as a phosphodiester bond (to produce longer contiguous subsequence), a nucleotidic linker of any desirable length, or any combinations thereof.

[00140] In such embodiments, the nucleic acid label of the detection reagent can be adapted to any configuration or structure. In some embodiments, the nucleic acid label can be single-stranded, double-stranded, partially double-stranded, a hairpin, linear, circular, branched, a concatemer, or any combinations thereof. In some embodiments, the nucleic acid label can be a linear polynucleotide sequence.

[00141] In some embodiments, the nucleic acid label can be a circular polynucleotide sequence. The advantage of using a circular nucleic acid label is that it can be amplified using rolling-circle amplification or hyperbranched rolling-circle amplification to generate a long continuous nucleic acid molecule that contains multiple copies of the same nucleic acid label sequences linked in series (also known as concatemer), thus resulting in an amplified signal. In some embodiments, instead of pre-forming the detection reagents comprising the circular nucleic acid label(s) and the probe reagent(s), detection reagents comprising linear polynucleotide(s) and the probe reagent(s) can be first synthesized. The circular nucleic acid labels can then added to hybridize with the linear polynucleotide(s) before or after the probe reagent(s) bind to the analytes. In such embodiments, while requiring an extra step, this approach can have the

advantage over direct attachment of circular nucleic acid labels in that it does not require chemical modification of the nucleic acid label for conjugation with the probe reagents, thus resulting in a circular nucleic acid label that is compatible with a broader range of amplification enzymes. Furthermore, the linear polynucleotides can be smaller than the secondary circular nucleic acid labels, facilitating diffusion of the probe reagents (and the detection reagents) to their targets. In other embodiments, the linear polynucleotides can be circularized using a suitable double-stranded or single-stranded ligase, with or without the addition of a suitable ligation template. Without wishing to be bound by theory, such embodiments can avoid the extra hybridization that is required when a pre-circularized oligonucleotide is used instead.

[00142] When the detection reagents described herein are used as FISH probes, the nucleic acid labels and the FISH probes can be part of the same nucleic acid sequence construct, and thus the circularization can encompass the entire construct (e.g., both the nucleic acid labels and FISH probes). A schematic representation of exemplary FISH probes for SeqTagged FISH is shown in **Fig. 13**.

[00143] In some embodiments, the method described herein can be used to identify a class an analyte, e.g., a pathogen belongs to. For example, the method can be used to identify if a pathogen is a Gram-negative, Gram-positive, or some other class of pathogen, e.g., yeast. Thus, the method described herein can be used to as Gram test to determine whether a suspected pathogen is Gram positive, Gram negative or yeast. This can be useful for quickly identifying the type of infection in a subject and administering appropriate therapy. By way of example only, this can be accomplished using a detection reagent comprising a class-specific probe, also referred to as “Gram-stain like probe” herein. Again by way of example only, the probe can be a DNA probe for FISH.

[00144] In some embodiment, the FISH probe for identifying eubacteria can comprise the nucleotide sequence of SEQ ID NO: 4 (GCTGCCTCCCGTAGGAGT). An exemplary SeqTag labeled FISH-probe for identifying eubacteria can comprise the nucleotide sequence of SEQ ID NO: 5 (CTGCCTCCCGTAGGAGTTTTTCGCTTTAGCCTAAGTGAAATC).

[00145] In some embodiments, the FISH probe for identifying yeast can comprise the nucleotide sequence of SEQ ID NO: 6 (CTCTGGCTTCACCCTATTC. An exemplary SeqTag labeled FISH-probe for identifying yeast can comprise the nucleotide sequence of SEQ ID NO: 7 (CTCTGGCTTCACCCTATTCTTTTTTCGCTTTTTTGGGGAAAAGACA).

[00146] In some embodiments, the FISH probe for identifying firmicutes can comprise the nucleotide sequence of SEQ ID NO: 8 (CGGAAGATTCCCTACTGC). An exemplary SeqTag labeled FISH-probe for identifying yeast can comprise the nucleotide sequence of SEQ ID NO: 9 (CGGAAGATTCCCTACTGCTTTTTCGCTTTCTGTAATGGAGTGGA).

[00147] In the SeqTag labeled FISH probes discussed above, each probe can be assigned a particular “signal signature” for each of the three readout steps. For example, colors can be labeled at B, C, and D. In some embodiments, each color can be from a different fluorophore, such as FAM, Cy3 and Cy5. Each signal signature can take advantage of multiple fluorophores simultaneously or even the same fluorophore multiple times, e.g., a SeqTag with signal signature corresponding to “DDDC.”

[00148] An exemplary signal signature for identifying eubacteria, yeast or firmicutes is shown in **Table 1**. As shown, each class has a different assigned code for the first readout step. For example, for the first readout, eubacteria are assigned color C, yeast color D and firmicutes color B. On readout, eubacteria will show up as color C, yeast as color D, firmicutes as both colors B and C.

Table 1

Name	Assigned code			Effective code		
	S1	S2	S3	S1	S2	S3
Eubacteria-Kempf	C			C		
All_yeast-Kempf	D			D		
Firmicutes-pB-00196	B			BC		

[00149] After identifying the class of pathogens, the pathogens can be further probed for identifying the specific pathogen or genus using the method described herein. For example, the detection reagent can comprise a pathogen specific probe that binds to a specific pathogen or genus of pathogens. For example, the probe can be a FISH probe that specifically binds to a specific pathogen. Some exemplary pathogen specific FISH probes are shown in **Table 2**.

Table 2

Name		probeBase FISH Sequence	Assigned code	Effective code
SEQ ID NO:	Gram +			
10	Staph_spp-Kempf	TCCTCCATATCTCTGCGC	DD B	BC DD B
11	S_Aureus-Kempf	GAAGCAAGCTTCTCGTCCG	CD B	BC CDDD BB
12	Streptococcus_spp-	CACTCTCCCCTTCTGCAC	DD D	BC DD D

	Kempf			
13	S_Pneumoniae-Kempf	GTGATGCAAGTGCACCTT	B DC	BC DDB DDC
14	S_Pyogenes-Kempf	TTCCAAAGCGTACATTGGTT	C DB	BC DDC DDB
15	S_Agalactiae-Kempf	GTAAACACCAAACMTCAGCG	D DC	BC DDD DDC
16	B_subtilis-pB-00401	CGA AGG GGA CGT CCT ATC T	C DD	BC C DD
17	L_acidophilus-pB-00711	CAG GCT TGC TCC TCG TTG	DD C	BC DD C
Gram -				
18	P_Aeruginosa-Kempf	TCTCGGCCTTGAAACCCC	B B	C B B
19	K_Pneumoniae-Kempf	CCTACACACCAGCGTGCC	B DD	C B DD
20	H_influenzae-pB-00348	CCG CAC TTT CAT CTT CCG	B BC	C B BC
21	B_cepacia-pB-00346	CTG TGC GCC GGT TCT CTT	DD B	C DD B
22	K_oxytoca-pB-01681	CTA CAA GAC TCC AGC CTG CC	DD C	C DD C
23	E_coli-pB-02569-compl	ATG AGC AAA GGT ATT AAC TTT ACT CCC	B C	C B C
24	Shewanella_spp-pB-01191-mod	AGC TAA TCC CAC CTA GGT TCA TC	BC B	C BC B
25	H_pylori-pB-00361	CACACCTGACTGACTATCCCCG	C B	C C B
Yeast				
26	C_Albigans-Kempf	GCCAAGGCTTATACTCGCT	C BC	D C BC
27	C_Glabrata_Kempf	CCGCCAAGCCACAAGGACT	C CD	D C CD
28	C_Krusei-Kempf	GATTCTCGGCCCCATGGG	BC C	D BC C
29	C_Parapsilosis-Kempf	CCTGGTTCGCCAAAAGGC	CD C	D CD C

[00150] Exemplary SeqTag labeled FISH-probe for identifying a specific pathogen are shown in **Table 3**. As shown in **Table 3**, each specific pathogen can be assigned a specific signal signature based on an assigned color for each readout step and identity of a pathogen can be decoded using this table when the method is carried out using this set of assigned coding.

Table 3

SEQ ID NO:	Name	SeqTag-labeled FISH-probe Sequence
		Gram +
30	Staph_spp-Kempf	TCCTCCATATCTCTGCGCTTTTTCGCTTTCTGGAGAAAGGGCCATTTT TCGCTTTCGGTTCCAAAGACACTTTTTCGCTTTCTGGAGAAAGGGCC A
31	S_Aureus-Kempf	GAAGCAAGCTTCTCGTCCGTTTTTCGCTTTCTGGAGAAAGGGCCATT TTTCGCTTTCGGTTCCAAAGACACTTTTTCGCTTTGGAAGCACCTAT TCC
32	Streptococcus_spp-Kempf	CACTCTCCCCCTCTGCACTTTTTCGCTTTCTGGAGAAAGGGCCATTTT TCGCTTTTCACGATCCCATGTATTTTTTCGCTTTCTGGAGAAAGGGCC A
33	S_Pneumoniae-Kempf	GTGATGCAAGTGCACCTTTTTTTCGCTTTGAAGCCGGTTATAGCTTT TTCGCTTTTAGGCATTAGCATTGTTTTTCGCTTTTCACGATCCCATGT A
34	S_Pyogenes-Kempf	TTCCAAAGCGTACATTGGTTTTTTTTTCGCTTTCGGTTCCAAAGACACT TTTTTCGCTTTGGAAGCACCTATTCCTTTTTTCGCTTTTCACGATCCCAT GTA
35	S_Agalactiae-Kempf	GTAAACACCAAACMTCAGCGTTTTTCGCTTTGAAGCCGGTTATAGC TTTTTCGCTTTCTGGAGAAAGGGCCATTTTTTCGCTTTTCACGATCCC ATGTA
36	B_subtilis-pB-00401	CGA AGG GGA CGT CCT ATC TTTTTTTCGCTTTTCACGATCCCATGTATTTTTTCGCTTTGGAAGCACCT ATTCCTTTTTTCGCTTTTCACGATCCCATGTA
37	L_acidophilus-pB-00711	CAG GCT TGC TCC TCG TTGTTTTTCGCTTTCTGGAGAAAGGGCCATTTTTTCGCTTTGAAGCCG GTTATAGCTTTTTTCGCTTTCTGGAGAAAGGGCCA
Gram -		
38	P_Aeruginosa-Kempf	TCTCGGCCTTGAAACCCCTTTTTTCGCTTTTAGGCATTAGCATTGTTTT TCGCTTTCGGTTCCAAAGACAC
39	K_Pneumoniae-Kempf	CCTACACACCAGCGTGCCTTTTTCGCTTTTCACGATCCCATGTATTTT TCGCTTTTAGGCATTAGCATTGTTTTTCGCTTTTCACGATCCCATGTA
40	H_influenzae-pB-00348	CCG CAC TTT CAT CTT CCGTTTTTCGCTTTCGGTTCCAAAGACACTTTTTTCGCTTTTAGGCATT AGCATTGTTTTTCGCTTTGAAGCCGGTTATAGC
41	B_cepacia-pB-00346	CTG TGC GCC GGT TCT CTTTTTTTCGCTTTCTGGAGAAAGGGCCATTTTTTCGCTTTCGGTTCCA AAGACACTTTTTTCGCTTTCTGGAGAAAGGGCCA
42	K_oxytoca-pB-01681	CTA CAA GAC TCC AGC CTG CCTTTTTTCGCTTTCTGGAGAAAGGGCCATTTTTTCGCTTTGAAGCCGG TTATAGCTTTTTTCGCTTTCTGGAGAAAGGGCCA
43	E_coli-pB-02569-compl	ATG AGC AAA GGT ATT AAC TTT ACT CCCTTTTTTCGCTTTTAGGCATTAGCATTGTTTTTCGCTTTGAAGCCGG TTATAGC
44	Shewanella_spp-pB-01191-mod	AGC TAA TCC CAC CTA GGT TCA TCTTTTTTCGCTTTTAGGCATTAGCATTGTTTTTCGCTTTCGGTTCCAA AGACACTTTTTTCGCTTTGGAAGCACCTATTCC

45	H_pylori-pB-00361	CACACCTGACTGACTATCCCGTTTTTCGCTTTGGAAGCACCTATTCC TTTTTCGCTTTCGGTTCCAAAGACAC
		Yeast
46	C_Albigans-Kempf	GCCAAGGCTTATACTCGCTTTTTTCGCTTTCGGTTCCAAAGACACTT TTCGCTTTGGAAGCACCTATTCTTTTTTCGCTTTGAAGCCGGTTATA GC
47	C_Glabrata_Kempf	CCGCCAAGCCACAAGGACTTTTTTCGCTTTGAAGCCGGTTATAGCTT TTCGCTTTGGAAGCACCTATTCTTTTTTCGCTTTTCACGATCCCATG TA
48	C_Krusei-Kempf	GATTCTCGGCCCCATGGGTTTTTCGCTTTTAGGCATTAGCATTGTTTT TCGCTTTGAAGCCGGTTATAGCTTTTTTCGCTTTGGAAGCACCTATTC C
49	C_Parapsilosis-Kempf	CCTGGTTCGCCAAAAAGGCTTTTTTCGCTTTCTGGAGAAAGGGCCATT TTTTTCGCTTTGAAGCCGGTTATAGCTTTTTTCGCTTTGGAAGCACCTAT TCC

[00151] As shown in **Table 3**, the “signal signature” can comprise multiple fluorescence “colors” in each step. For example, *E. coli* can be marked as red in step 1 and red+green in step 2 and blue in step 3. The “signal signature” can also be encoded in the brightness of the color at each step. For example, *E. coli* could be marked as red in step 1 and 3 times brighter red in step 2.

[00152] Exemplary hybridization sites for generating the different signal signatures at different steps using the exemplary SeqTag labeled FISH-probes described above are shown in **Table 4**.

Table 4

SEQ ID NO:	Assigned “Color”	
		Set1
50	B	CGCTTTCTGTAATGGAGTGGA
51	C	CGCTTTAGCCTAAGTGAAATC
52	D	CGCTTTTTTGGGGAAAAGACA
		Set 2
53	B	CGCTTTTAGGCATTAGCATTG
54	C	CGCTTTGGAAGCACCTATTCC
55	D	CGCTTTCTGGAGAAAGGGCCA
		Set 3
56	B	CGCTTTCGGTTCCAAAGACAC
57	C	CGCTTTGAAGCCGGTTATAGC
58	D	CGCTTTTCACGATCCCATGTA

[00153] In some embodiments, multiple detection reagents can be used to identify a specific analyte. For example, a first set of probes can be used to identify whether the potential pathogen is gram-positive, gram-negative and then another set of probes to identify it more specifically. Both probes would need to produce a detectable signal, allowing one to use, for example, **Table 3** to uniquely identify the pathogen.

[00154] In some embodiments, the nucleic acid label can be a concatemer, including a rolon or a DNA nanoball that is large enough to act as a particle rather than simply a strand of nucleic acid. Using a concatemer as a nucleic acid label can eliminate the need for in-situ enzymatic treatment of circular nucleic acid amplification, but it can also increase the overall molecular weight of the detection reagent and thus retard diffusion. Similar to the circular nucleic acid labels as described above, an exemplary approach of hybridizing concatemers with the linear polypeptides of the detection reagents after probe reagents bind to the analytes can be used to facilitate diffusion of the probe reagents (and the detection reagents) to their targets.

[00155] To increase the accuracy and/or specificity of the methods described herein, in various embodiments, the nucleic acid label can be designed for minimal cross-hybridization of bases with each other. Various art-recognized computational programs or algorithms are available to design nucleic acid sequences with minimal cross-hybridization. Thus, one of skill in the art can optimize the nucleic acid label sequence using any methods or algorithms known in the art.

[00156] In some embodiments, the nucleic acid labels described herein can be synthetic nucleic acid molecules (e.g., DNA, RNA, or DNA/RNA hybrids), and can be rationally-designed to have features that optimize labeling and detection of the detection reagents, and that prevent secondary structure formation. In some embodiments, a nucleic acid label is a designed polynucleotide sequence from about 50 to 50,000 bases long.

[00157] In some embodiments, the nucleic acid labels described herein can be designed to minimize predictable secondary structures, and/or be designed such that each nucleic acid label can hybridize only against its own target. In some embodiments, the nucleic acid labels described herein can be designed to be devoid of any secondary structure. Putative secondary structures (e.g. hairpins, folding, or internal base pairing) can be predicted by methods known in the art such as MFOLD. Without intending to be limited to any theory, in some embodiments, predictable secondary structure in the nucleic acid label can be minimized by avoiding inverted

repeats and by skewing the backbone-specific content such that the backbone of the nucleic acid label is CT or GA-rich. Any art-recognized methods, e.g., MFOLD, can be used to verify if each nucleic acid label can hybridize only against its own target.

[00158] Sequences can also be screened to avoid common six-base-cutter restriction enzyme recognition sites. Selected sequences can be additionally subjected to predicted secondary structure analysis, and those with the least secondary structure may be chosen for further evaluation. Any program known in the art can be used to predict secondary structure, such as the MFOLD program (Zuker, 2003, *Nucleic Acids Res.* 31 (13):3406-15; Mathews et al., 1999, *J. Mol. Biol.* 288:911-940).

[00159] In some embodiments, the nucleic acid label can comprise only a subset of the A, G, C, T (and/or U) nucleotides or modified nucleotides thereof. In some embodiments, the nucleic acid label can comprise only one of the A, G, C, T (and/or U) nucleotides or modified nucleotides thereof. In some embodiments, the nucleic acid can comprise two of the A, C, T (and/or U) nucleotides or modified nucleotides thereof. In some embodiments, the nucleic acid label can comprise only three of the A, G, C, and T (and/or U) nucleotides or modified nucleotides thereof. In some embodiments, the nucleic acid label can comprise only four of the A, G, C, and T (and/or U) nucleotides or modified nucleotides thereof. The subset of the A, G, C and T (and/or U) nucleotides or modified nucleotide thereof can be selected from the group consisting of A; G; C; T; U; (A,G); (A,C); (G,T); (G,U); (C,T); (C,U); (G,T,U); (C,T,U); (A,C,G); (A,G,T); (A,G,U); (A,C,T); (A,C,U); (C,G,T); (C,G,U); (A,C,T,U); and (C,G,T,U).

[00160] Without wishing to be bound, the nucleic acid label can be a modified nucleic acid label. An exemplary modification of the nucleic acid label includes, without limitations, attaching one or more detectable molecules to the nucleic acid label (either to one end of or along the nucleic acid label sequence). The detectable molecule can be any optical molecule, including, but not limited to, a small-molecule dye, a fluorescent protein, a quantum dot, or any combinations thereof. In another embodiment, at least one end of the nucleic acid label can be modified to include a chemical functional group and/or a protein or peptide to facilitate the conjugation between the nucleic acid label and the probe reagent.

[00161] In some embodiments, at least one (including, e.g., at least two, at least three, at least four, at least five, at least six or more) nucleic acids or nucleotides present in the nucleic acid label can be modified. For example, the nucleic acid can comprise one or more nucleic acid

modifications as described herein, e.g., selected from the group consisting of internucleotide linkage modifications (intersugar linkage modifications), sugar modifications, nucleobase modifications, backbone modifications/replacements, and any combinations thereof.

Conjugation between a nucleic acid label and a probe reagent

[00162] In accordance with embodiments of various aspects described herein, the detection reagent comprises at least one probe reagent and at least one nucleic acid label, wherein the probe reagent and the nucleic acid label can be conjugated together by any methods known in the art.

[00163] In some embodiments, the probe reagent and the nucleic acid label can be attached or conjugated together by a linker. As used herein, the term “linker” generally refers to an entity that connects the probe reagent and the nucleic acid label together. The linker can be monovalent or multivalent. The term “monovalent” as used herein refers to the capacity of a linker to join one probe reagent to one nucleic acid label. The term “multivalent” as used herein refers to the capacity of a linker to bind with one or more probe reagents and/or nucleic acid labels. In some embodiments, a multivalent linker can join at least one probe reagent to a plurality of nucleic acid labels (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10 or more nucleic acid labels).

[00164] In some embodiments, the term “linker” means an organic moiety that connects two parts of a compound. Such linkers typically comprise a direct bond or an atom such as oxygen or sulfur, a unit such as NH, C(O), C(O)NH, SO, SO₂, SO₂NH, SS, or a chain of atoms, such as substituted or unsubstituted C₁-C₆ alkyl, substituted or unsubstituted C₂-C₆ alkenyl, substituted or unsubstituted C₂-C₆ alkynyl, substituted or unsubstituted C₆-C₁₂ aryl, substituted or unsubstituted C₅-C₁₂ heteroaryl, substituted or unsubstituted C₅-C₁₂ heterocyclyl, substituted or unsubstituted C₃-C₁₂ cycloalkyl, where one or more methylenes can be interrupted or terminated by O, S, S(O), SO₂, NH, C(O).

[00165] In some embodiments, the linker is a branched linker. The branchpoint of the branched linker may be at least trivalent, but can be a tetravalent, pentavalent or hexavalent atom, or a group presenting such multiple valencies. In some embodiments, the branchpoint is -N, -N(R)-C, -O-C, -S-C, -SS-C, -C(O)N(R)-C, -OC(O)N(R)-C, -N(R)C(O)-C, or -N(R)C(O)O-C; wherein R is independently for each occurrence H or optionally substituted alkyl. In some embodiments, the branchpoint is glycerol or derivative thereof.

[00166] In some embodiments, linker comprises a cleavable linking group. As used herein, a “cleavable linking group” is a chemical moiety which is sufficiently stable outside the cell, but which upon entry into a target cell is cleaved to release the two parts the linker is holding together. In a preferred embodiment, the cleavable linking group is cleaved at least 10 times or more, preferably at least 100 times faster in the target cell or under a first reference condition (which can, e.g., be selected to mimic or represent intracellular conditions) than in the blood or serum of a subject, or under a second reference condition (which can, e.g., be selected to mimic or represent conditions found in the blood or serum).

[00167] Cleavable linking groups are susceptible to cleavage agents, e.g., pH, redox potential or the presence of degradative molecules. Generally, cleavage agents are more prevalent or found at higher levels or activities inside cells than in serum or blood. Examples of such degradative agents include: redox agents which are selected for particular substrates or which have no substrate specificity, including, e.g., oxidative or reductive enzymes or reductive agents such as mercaptans, present in cells, that can degrade a redox cleavable linking group by reduction; esterases; amidases; endosomes or agents that can create an acidic environment, e.g., those that result in a pH of five or lower; enzymes that can hydrolyze or degrade an acid cleavable linking group by acting as a general acid, peptidases (which can be substrate specific) and proteases, and phosphatases.

[00168] A linker can include a cleavable linking group that is cleavable by a particular enzyme. The type of cleavable linking group incorporated into a linker can depend on the cell to be targeted. For example, for liver targeting, cleavable linking groups can include an ester group. Liver cells are rich in esterases, and therefore the linker will be cleaved more efficiently in liver cells than in cell types that are not esterase-rich. Other cell-types rich in esterases include cells of the lung, renal cortex, and testis.

[00169] Linkers that contain peptide bonds can be used when targeting cell types rich in peptidases, such as liver cells and synoviocytes.

[00170] In some embodiments, cleavable linking group is cleaved at least 1.25, 1.5, 1.75, 2, 3, 4, 5, 10, 25, 50, or 100 times faster in the cell (or under in vitro conditions selected to mimic intracellular conditions) as compared to blood or serum (or under in vitro conditions selected to mimic extracellular conditions). In some embodiments, the cleavable linking group is cleaved by less than 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, or 1% in the blood (or in

vitro conditions selected to mimic extracellular conditions) as compared to in the cell (or under in vitro conditions selected to mimic intracellular conditions).

[00171] Exemplary cleavable linking groups include, but are not limited to, redox cleavable linking groups (e.g., -S-S- and -C(R)₂-S-S-, wherein R is H or C₁-C₆ alkyl and at least one R is C₁-C₆ alkyl such as CH₃ or CH₂CH₃); phosphate-based cleavable linking groups (e.g., -O-P(O)(OR)-O-, -O-P(S)(OR)-O-, -O-P(S)(SR)-O-, -S-P(O)(OR)-O-, -O-P(O)(OR)-S-, -S-P(O)(OR)-S-, -O-P(S)(ORk)-S-, -S-P(S)(OR)-O-, -O-P(O)(R)-O-, -O-P(S)(R)-O-, -S-P(O)(R)-O-, -S-P(S)(R)-O-, -S-P(O)(R)-S-, -O-P(S)(R)-S-, . -O-P(O)(OH)-O-, -O-P(S)(OH)-O-, -O-P(S)(SH)-O-, -S-P(O)(OH)-O-, -O-P(O)(OH)-S-, -S-P(O)(OH)-S-, -O-P(S)(OH)-S-, -S-P(S)(OH)-O-, -O-P(O)(H)-O-, -O-P(S)(H)-O-, -S-P(O)(H)-O-, -S-P(S)(H)-O-, -S-P(O)(H)-S-, and -O-P(S)(H)-S-, wherein R is optionally substituted linear or branched C₁-C₁₀ alkyl); acid cleavable linking groups (e.g., hydrazones, esters, and esters of amino acids, -C=NN- and -OC(O)-); ester-based cleavable linking groups (e.g., -C(O)O-); peptide-based cleavable linking groups, (e.g., linking groups that are cleaved by enzymes such as peptidases and proteases in cells, e.g., -NHCHR^AC(O)NHCHR^BC(O)-, where R^A and R^B are the R groups of the two adjacent amino acids). A peptide based cleavable linking group comprises two or more amino acids. In some embodiments, the peptide-based cleavage linkage comprises the amino acid sequence that is the substrate for a peptidase or a protease found in cells.

[00172] In some embodiments, an acid cleavable linking group is cleavable in an acidic environment with a pH of about 6.5 or lower (e.g., about 6.0, 5.5, 5.0, or lower), or by agents such as enzymes that can act as a general acid.

[00173] In addition to covalent linkages, two parts of a compound can be linked together by an affinity binding pair. The term “affinity binding pair” or “binding pair” refers to first and second molecules that specifically bind to each other. One member of the binding pair is conjugated with first part to be linked while the second member is conjugated with the second part to be linked. As used herein, the term “specific binding” refers to binding of the first member of the binding pair to the second member of the binding pair with greater affinity and specificity than to other molecules.

[00174] Exemplary binding pairs include any haptenic or antigenic compound in combination with a corresponding antibody or binding portion or fragment thereof (e.g., digoxigenin and anti-digoxigenin; mouse immunoglobulin and goat antimouse immunoglobulin) and

nonimmunological binding pairs (e.g., biotin-avidin, biotin-streptavidin, biotin-neutravidin, hormone [e.g., thyroxine and cortisol-hormone binding protein, receptor-receptor agonist, receptor-receptor antagonist (e.g., acetylcholine receptor-acetylcholine or an analog thereof), IgG-protein A, IgG-protein G, IgG-synthesized protein AG, lectin-carbohydrate, enzyme-enzyme cofactor, enzyme-enzyme inhibitor, and complementary oligonucleotide pairs capable of forming nucleic acid duplexes), and the like. The binding pair can also include a first molecule which is negatively charged and a second molecule which is positively charged.

[00175] One example of using binding pair conjugation is the biotin-avidin, biotin-streptavidin or biotin-neutravidin conjugation. In this approach, one of the molecule or the peptide is biotinylated and the other is conjugated with avidin or streptavidin. Many commercial kits are also available for biotinylating molecules, such as proteins.

[00176] Another example of using binding pair conjugation is the biotin-sandwich method. *See, e.g.,* example Davis et al., Proc. Natl. Acad. Sci. USA, 103: 8155-60 (2006). The two molecules to be conjugated together are biotinylated and then conjugated together using at least one tetravalent avidin-like molecule (e.g., avidin, streptavidin, or neutravidin) as a linker.

[00177] Accordingly, in some embodiments, both the nucleic acid label(s) and probe reagent(s) can be biotinylated and then linked together using an avidin-like molecule (e.g., avidin, streptavidin, or neutravidin). In one embodiment, neutravidin and/or streptavidin is used as a linker to bridge together the biotinylated nucleic acid label(s), e.g., DNA sequence(s), and biotinylated probe reagent(s), e.g., antibody. Without wishing to be bound by theory, each avidin-like molecule (e.g., avidin, streptavidin, or neutravidin) generally has four binding sites, so at most four molecules can be linked together. For example, one biotinylated probe reagent can be linked, via an avidin-like molecule (e.g., avidin, streptavidin or neutravidin), to three biotinylated nucleic acid labels, or in any other combinations (e.g., two biotinylated probe reagents linked to two biotinylated nucleic acid labels).

[00178] Biotinylation of a nucleic acid label (i.e., attaching a biotin to a nucleic acid label) can occur at any location of the nucleic acid label. In some embodiments, the nucleic acid label can be synthesized or modified with a terminal biotin, i.e., the nucleic acid label can have a biotin at its 5' end and/or 3' end. In other embodiments, the nucleic acid label can be synthesized or modified with an internal biotin, i.e., the nucleic acid label can have a biotin anywhere between its 5' and 3' ends, but not at its 5' and/or 3' ends. Such internal biotinylation can leave at least

one end (e.g., both ends) of the nucleic acid label accessible, allowing the nucleic acid label to be circularized after the nucleic acid label has bound, which can in turn, for example, enable rolling circle amplification as described earlier. Chemical modification of the nucleic acid label, e.g., to attach a terminal and/or an internal biotin to the nucleic acid label after synthesis, is within one of skill in the art.

[00179] For some embodiments where the probe reagent is a nucleic acid, an example of using binding pair conjugation is double-stranded nucleic acid conjugation. In this approach, the first part to be linked is conjugated with a first strand of the double-stranded nucleic acid and the second part to be linked is conjugated with the second strand of the double-stranded nucleic acid. Nucleic acids can include, without limitation, defined sequence segments and sequences comprising nucleotides, ribonucleotides, deoxyribonucleotides, nucleotide analogs, modified nucleotides and nucleotides comprising backbone modifications, branchpoints and nonnucleotide residues, groups or bridges.

[00180] In some embodiments, the linker can be a linker molecule. Examples of linker molecules can include, but are not limited to, a polymer, sugar, nucleic acid, peptide, protein, hydrocarbon, lipid, polyethylene glycol, crosslinker, or combination thereof.

[00181] Non-limiting examples of crosslinkers that can be used as linker molecules can include, but are not limited to, amine-to-amine crosslinkers (e.g., but are not limited to the ones based on NHS-ester and/or imidoester reactive groups), amine-to-sulfhydryl crosslinkers, carboxyl-to-amine crosslinkers (e.g., but are not limited to, carbodiimide crosslinking agents such as DCC, and/or EDC (EDAC); and/or N-hydroxysuccinimide (NHS)), photoreactive crosslinkers (e.g., but not limited to, aryl azide, diazirine and any art-recognized photo-reactive (light-activated) chemical crosslinking reagents), sulfhydryl-to-carbohydrate crosslinkers (e.g., but are not limited to the ones based on maleimide and/or hydrazide reactive groups), sulfhydryl-to-hydroxyl crosslinkers (e.g., but are not limited to the ones based on maleimide and/or isocyanate reactive groups), sulfhydryl-to-sulfhydryl crosslinkers (e.g., but are not limited to, maleimide and/or pyridyldithiol reactive groups), sulfo-SMCC crosslinkers, sulfo-SBED biotin label transfer reagents, sulfhydryl-based biotin label transfer reagents, photoreactive amino acids (e.g., but are not limited to diazirine analogs of leucine and/or methionine), NHS-azide Staudinger ligation reagents (e.g., but are not limited to, activated azido compounds), NHS-phosphine Staudinger ligation reagents (e.g., but are not limited to, activated phosphine

compounds), and any combinations thereof.

[00182] In some embodiments, any commercially available crosslinkers (e.g., but not limited to the ones from Thermo Scientific or Piercenet, Rockford, IL) can be used as a linker molecule herein.

[00183] In some embodiments, the term “linker” as used herein can be a physical substrate. In some embodiments, the physical substrate is a particle. The particle can be of any shape, e.g., spheres; rods; shells; beads, tubes, wires, and prisms; and these particles can be part of a network.

[00184] The particles can be made of any materials. In some embodiments, the particle can comprise a material selected from the group consisting of metal (e.g., gold, or iron), metal oxides (e.g., iron oxide), plastic, glass, polymer (e.g., polystyrene), and any combinations thereof.

[00185] For in vivo purposes, e.g., disease and/or pathogen diagnosis and/or targeted drug delivery, the polymer can be biocompatible and/or biodegradable. The term “biocompatible polymer” refers to polymers which, in the amounts employed, are non-toxic and substantially non-immunogenic when used internally in the patient and which are substantially insoluble in the body fluid of the mammal. Examples of non-biodegradable biocompatible polymers include, by way of example, cellulose acetates (including cellulose diacetate), ethylene vinyl alcohol copolymers, hydrogels (e.g., acrylics), polyacrylonitrile, polyvinylacetate, cellulose acetate butyrate, nitrocellulose, copolymers of urethane/carbonate, copolymers of styrene/maleic acid, and any combinations thereof. Biodegradable polymers are known in the art, e.g., without limitations, linear-chain polymers such as polylactides, polyglycolides, polycaprolactones, polyanhydrides, polyamides, polyurethanes, polyesteramides, polyorthoesters, polydioxanones, polyacetals, polyketals, polycarbonates, polyorthocarbonates, polyphosphazenes, polyhydroxybutyrates, polyhydroxyvalerates, polyalkylene oxalates, polyalkylene succinates, poly(malic acid), poly(amino acids), polyvinylpyrrolidone, polyethylene glycol, polyhydroxycellulose, chitin, chitosa, and copolymers, terpolymers and any combinations thereof. Other biodegradable polymers include, for example, fibrin, gelatin, collagen, and any combinations thereof.

[00186] The particles can be of any size for the purpose of various aspects described herein, provided that the particle size does not significantly affect the diffusion property of the detection reagent. For example, the particle size can range from 5 nm to 1 mm, from about 10 nm to about

500 μm , or from about 50 nm to about 250 μm . In some embodiments, a particle described herein is a nanoparticle or a microparticle. As used herein, the term “nanoparticle” refers to particles that are on the order of 10^{-9} or one billionth of a meter and below 10^{-6} or 1 millionth of a meter in size. The term “microparticle” as used herein refers to particles that are on the order of 10^{-6} or one millionth of a meter and below 10^{-3} or 1 thousandth of a meter in size. In some embodiments, the particle can be selected from a group consisting of a gold nanoparticle or microparticle, a paramagnetic nanoparticle or microparticle, a magnetic nanoparticle or microparticle, a polystyrene nanoparticle or microparticle, a nanotube or a microtube, a nanowire or a microwire, and any combinations thereof.

[00187] The particles can be adapted to possess at least one additional property, depending on various applications. In some embodiments, the particles can be adapted to be magnetic responsive or paramagnetic-responsive, e.g., magnetic or paramagnetic particles. In some embodiments, the particles can be adapted to be a delivery vehicle. For example, in some embodiments, the particles can be encapsulated with a therapeutic agent, e.g., for targeted drug delivery to treat a disease or disorder.

[00188] In some embodiments, the particles can be modified. For example, the particles can be conjugated with proteins, peptides, nucleic acids, or any combinations thereof. In one embodiment, the particles can be surface-conjugated or coated with one member of the binding pair as described above (e.g., for biotin-streptavidin interaction, streptavidin-coated particles can be used for the purpose of various aspects described herein). In some embodiments, the particles can be surface-activated with functional groups (e.g., but not limited to, amine, carboxylic acid, epoxy, tosyl, silane, and any combinations thereof), e.g., to provide binding sites for probe reagents and/or nucleic acid labels. Methods for surface modifications of particles, such as nanoparticles or microparticles, are well known in the art. One of skill in the art can readily modify or activate the surface of particles using any art-recognized reactions, e.g., covalently through crosslinkers, or through protein interaction.

[00189] The particles described herein can be used in conjunction with the organic linker described above, to form a conjugate linker, or the particles can be used alone as a linker. Both the nucleic acid labels and the probe reagents can be coupled to the particles using a multitude of methods. These include, but are not limited to, direct covalent attachment such as using chemical crosslinkers based on chemistries such as NHS, maleimide, tosyl, isocyanide, etc.; chemical

linkage such as using EDC chemistry, thiol adsorption to gold, vinyl/acrylate radical reaction, acrylate-based addition (of e.g., thiols); and protein mediated couplings based on proteins such as streptavidin (and its relatives such as avidin, neutravidin, etc.), protein A, and secondary antibodies, and any combinations thereof.

[00190] The particles can act as hubs that facilitate a conjugation of multiple nucleic acid labels to single or multiple probes. Depending on applications and/or properties of nucleic acid labels and/or probe reagents, particles can be used as hubs for multi-conjugation. For example, in some embodiments, by acting as a hub, the particles can allow multiple nucleic acid labels to be present at each location (e.g., location of a target molecule or analyte) where the probe binds. Accordingly, the signal generated from the detection reagent can be amplified and thus greater than if only a single label was present. This is especially desirable where antigens/targets or analytes are sparse in a sample.

[00191] In some embodiments, the particles can allow multiple probes to be arranged in proximity to each other. Thus, the particles can allow several weaker binding events to combine into strong binding. This “avidity action” can transform probe reagents with individually weak affinities into effective sensors.

[00192] In some embodiments, by acting as a hub, the particles can allow each probe reagent to conjugate to multiple nucleic acid labels. This capability can eliminate the need for other amplification methods (e.g., rolling circle amplification) as multiple nucleic acid labels corresponding to a probe reagent can be used as a form of signal amplification. In some embodiments, this capability can also be used to separate the detection reagents with one nucleic acid label from the ones with different nucleic acid labels, which can, in turn, enable, for example, sequencing by single-base extension. Furthermore, different nucleic acid labels can be conjugated to the particles at controlled ratios, and the ratios can encode additional information. By way of example only, to capture in situ information of one or more enzymes’ behavior (e.g., relative enzyme concentration) in the presence of an analyte, two or more different nucleic acid labels can be conjugated to the particles, wherein a subpopulation of the nucleic acid labels corresponding to the probe reagent is not cleavable, while other subpopulations of the nucleic acid labels are each adapted to be cleavable in the presence of their respective enzymes. In such embodiments, the cleavable nucleic acid labels can be conjugated to the particles by cleavable peptide bonds specific for corresponding enzymes. By comparing the ratios of signals generated

from the various nucleic acid labels, one would be able to determine the relative enzyme concentrations in the presence of an analyte.

[00193] The arrangement of the probe reagent(s) and/or nucleic acid label(s) on the particles can vary with a number of factors, e.g., applications, properties of probe reagents and/or nucleic acid labels, sample properties, and/or analytes of interests. In some embodiments, the probe reagents and nucleic acid labels can be conjugated to the particle directly (see, e.g., **Fig. 4A**). In some embodiments, one component can be linked to the particle through the other. In such embodiments, the probe reagents can be conjugated or linked to the particle through the nucleic acid labels (see, e.g., **Fig. 4B**), e.g., to allow the probe reagents being more accessible by the corresponding analytes in a sample.

[00194] In some embodiments, the detection reagents can further comprise at least one substrate linker conjugated to the particle. In such embodiment, the substrate linker can allow the detection reagents to be immobilized to a solid support or substrate.

Detectable molecules or detectable labels

[00195] A detectable molecule or detectable label can be covalently attached to a decoder probe or complementary nucleobase before or after the decoder probe or the complementary nucleobase is attached to the pre-determined subsequences or hybridization sites of a nucleic acid label. For example, in attaching a detectable label to a decoder probe, the label can be covalently attached by incorporation of a nucleotide containing a detectable label into the nucleic acid during its synthesis, but before it is hybridized the pre-determined subsequence of the nucleic acid label. Alternatively, during the synthesis of a decoder probe sequence, a nucleotide containing a detectable label acceptor group can be included, and the detectable label can be added to the decoder probe after its synthesis, either before or after it is hybridized to the pre-determined subsequences of the nucleic acid label. Alternatively, the detectable label can be indirectly attached to the decoder probe, for example, by incorporating a nucleotide containing a ligand-binding molecule (e.g., biotin) into the decoder probe during synthesis, and by adding a ligand (e.g., streptavidin) that is covalently attached to the detectable molecule, or vice versa.

[00196] In some embodiments, the ratios of a detectable label to a decoder probe can range from about 1:1 to about 100:1, from 1:1 to about 50:1, from about 1:1 to about 25:1, from about

1:1 to about 10:1, or from about 1:1 to about 5:1. When a decoder probe comprises more than one detectable label, each detectable label can be attached to a nucleotide of the decoder probe.

[00197] A detectable label or a detectable molecule can be attached to any nucleotide including both natural and non-natural nucleotides. A nucleotide contains three parts, a phosphate group, a pentose five-carbon sugar molecule, and an organic base. In RNA, the pentose is ribose and in DNA it is deoxyribose and so nucleotides for incorporation into RNA are called ribonucleotides and nucleotides for incorporation into DNA are called deoxyribonucleotides. Three bases adenine, guanine, and cytosine are found in both DNA and RNA while thymine is normally found only in DNA and uracil is normally found only in RNA. Nucleotides can have one, two or three attached phosphate groups and are sometimes referred to as nucleoside phosphates. Nucleotides can contain modified nucleosides having modified bases (e.g., 5-methyl cytosine) and modified sugar groups (e.g., 2'O-methyl ribosyl, 2'O-methoxyethyl ribosyl, 2'fluoro ribosyl, 2'amino ribosyl, and the like). An example of non-natural bases that are used in the art are isocytidine and isoguanine.

[00198] A detectable label or a detectable molecule as used herein is intended to mean an individual measurable moiety, such as a radioisotope, fluorochrome, dye, enzyme (including its effect on a substrate), nanoparticle, chemiluminescent marker, biotin, or other moiety known in the art that is measurable by analytical methods. A detectable label or a detectable molecule can be attached to a nucleotide using methods well known in the art and exemplified herein.

[00199] Without limitations, examples of a detectable label or a detectable molecule that can be utilized by some aspects described herein can include optical reporters or optical labels. Suitable optical reporters or optical labels include, but are not limited to, fluorescent reporters and chemiluminescent groups. A wide variety of fluorescent reporter dyes are known in the art. Typically, the fluorophore is an aromatic or heteroaromatic compound and can be a pyrene, anthracene, naphthalene, acridine, stilbene, indole, benzindole, oxazole, thiazole, benzothiazole, cyanine, carbocyanine, salicylate, anthranilate, coumarin, fluorescein, rhodamine or other like compound. Suitable fluorescent reporters include xanthene dyes, such as fluorescein or rhodamine dyes, including, but not limited to, Alexa Fluor® dyes (InvitrogenCorp.; Carlsbad, Calif), fluorescein, fluorescein isothiocyanate (FITC), Oregon Green™, rhodamine, Texas red, tetra-rhodamine isothiocyanate (TRITC), 5-carboxyfluorescein (FAM), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), tetrachlorofluorescein (TET), 6-carboxyrhodamine (R6G),

N,N,N,N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX). Suitable fluorescent reporters also include the naphthylamine dyes that have an amino group in the alpha or beta position. For example, naphthylamino compounds include 1-dimethylamino-naphthyl-5-sulfonate, 1-anilino-8-naphthalene sulfonate, 2-p-toluidinyl-6-naphthalene sulfonate, and 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS). Other fluorescent reporter dyes include coumarins, such as 3-phenyl-7-isocyanatocoumarin; acridines, such as 9-isothiocyanatoacridine and acridine orange; N-(p(2-benzoxazolyl)phenyl)maleimide; cyanines, such as Cy2, indodicarbocyanine 3 (Cy3), indodicarbocyanine 5 (Cy5), indodicarbocyanine 5.5 (Cy5.5), 3-(-carboxy-pentyl)-3'-ethyl-5,5'-dimethyloxacarbocyanine (CyA); 1H,5H,11H, 15H-Xantheno[2,3,4-ij:5,6,7-i'j']diquinolizin-18-ium, 9-[2(or 4)-[[[6-[2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl] amino]sulfonyl]-4(or 2)-sulfophenyl]-2,3,6,7,12,13,16,17octahydro-inner salt (TR or Texas Red); BODIPYTM dyes; benzoxadiazoles; stilbenes; pyrenes; and the like. Many suitable forms of these fluorescent compounds are available and can be used.

[00200] Examples of fluorescent proteins suitable for use as imaging agents include, but are not limited to, green fluorescent protein, red fluorescent protein (e.g., DsRed), yellow fluorescent protein, cyan fluorescent protein, blue fluorescent protein, and variants thereof (see, e.g., U.S. Pat. Nos. 6,403, 374, 6,800,733, and 7,157,566). Specific examples of GFP variants include, but are not limited to, enhanced GFP (EGFP), destabilized EGFP, the GFP variants described in Doan et al, *Mol. Microbiol.*, 55:1767-1781 (2005), the GFP variant described in Crameri et al, *Nat. Biotechnol.*, 14:315319 (1996), the cerulean fluorescent proteins described in Rizzo et al, *Nat. Biotechnol.*, 22:445 (2004) and Tsien, *Annu. Rev. Biochem.*, 67:509 (1998), and the yellow fluorescent protein described in Nagai et al, *Nat. Biotechnol.*, 20:87-90 (2002). DsRed variants are described in, e.g., Shaner et al, *Nat. Biotechnol.*, 22:1567-1572 (2004), and include mStrawberry, mCherry, morange, mBanana, mHoneydew, and mTangerine. Additional DsRed variants are described in, e.g., Wang et al, *Proc. Natl. Acad. Sci. U.S.A.*, 101:16745-16749 (2004) and include mRaspberry and mPlum. Further examples of DsRed variants include mRFPmars described in Fischer et al, *FEBS Lett.*, 577:227-232 (2004) and mRFPruby described in Fischer et al, *FEBS Lett.*, 580:2495-2502 (2006).

[00201] Amine-reactive and thiol-reactive fluorophores are available and generally used for labeling nucleotides and biomolecules. In some embodiments, nucleotides are fluorescently labeled during chemical synthesis, for example, incorporation of amines or thiols during

nucleotide synthesis permit addition of fluorophores. Fluorescently labeled nucleotides are commercially available. For example, uridine and deoxyuridine triphosphates are available that are conjugated to ten different fluorophores that cover the spectrum.

[00202] In some embodiments, radioisotopes can be utilized as detectable molecules or detectable molecules for labeling nucleotides including, for example, ^{32}P , ^{33}P , ^{35}S , ^3H , and ^{125}I . These radioisotopes have different half-lives, types of decay, and levels of energy which can be tailored to match the needs of a particular experiment. For example, ^3H is a low energy emitter which results in low background levels, however this low energy also results in long time periods for autoradiography. Radioactively labeled ribonucleotides and deoxyribonucleotides are commercially available. Nucleotides are available that are radioactively labeled at the first, or α , phosphate group, or the third, or γ , phosphate group. For example, both $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ and $[\gamma\text{-}^{32}\text{P}]\text{dATP}$ are commercially available. In addition, different specific activities for radioactively labeled nucleotides are also available commercially and can be tailored for different experiments.

[00203] Suitable non-metallic isotopes include, but are not limited to, ^{11}C , ^{14}C , ^{13}N , ^{18}F , ^{123}I , ^{124}I , and ^{125}I . Suitable radioisotopes include, but are not limited to, $^{99\text{m}}\text{Tc}$, ^{95}Tc , ^{111}In , ^{62}Cu , ^{64}Cu , Ga, ^{68}Ga , and ^{153}Gd . Suitable paramagnetic metal ions include, but are not limited to, Gd(III), Dy(III), Fe(III), and Mn(II). Suitable X-ray absorbers include, but are not limited to, Re, Sm, Ho, Lu, Pm, Y, Bi, Pd, Gd, La, Au, Au, Yb, Dy, Cu, Rh, Ag, and Ir. In some embodiments, the radionuclide is bound to a chelating agent or chelating agent-linker attached to the aggregate. Suitable radionuclides for direct conjugation include, without limitation, ^{18}F , ^{124}I , ^{125}I , ^{131}I , and mixtures thereof. Suitable radionuclides for use with a chelating agent include, without limitation, ^{47}Sc , ^{64}Cu , ^{67}Cu , ^{89}Sr , ^{86}Y , ^{87}Y , ^{90}Y , ^{105}Rh , ^{111}Ag , ^{111}In , ^{117}mSn , ^{149}Pm , ^{153}Sm , ^{166}Ho , ^{177}Lu , ^{186}Re , ^{188}Re , ^{211}At , ^{212}Bi , and mixtures thereof. Suitable chelating agents include, but are not limited to, DOTA, BAD, TETA, DTPA, EDTA, NTA, HDTA, their phosphonate analogs, and mixtures thereof. One of skill in the art will be familiar with methods for attaching radionuclides, chelating agents, and chelating agent-linkers to the particles.

[00204] Non-radioactive and non-fluorescent label monomers are also available. For example, biotin can be attached directly to nucleotides and detected by specific and high affinity binding to avidin or streptavidin which has been chemically coupled to an enzyme catalyzing a colorimetric reaction (such as phosphatase, luciferase, or peroxidase). Digoxigenin labeled

nucleotides can also similarly be used for non-isotopic detection of nucleic acids. Biotinylated and digoxigenin-labeled nucleotides are commercially available.

[00205] In some embodiments, enzymatic reaction on a substrate can be utilized a detection method. In such embodiments, enzymes (e.g., horseradish peroxidase or alkaline phosphatase) can be linked to a nucleotide of a nucleic acid label and/or decoder probe. During detection, a substrate on which the particular enzyme reacts can be added to induce a colorimetric reaction. Any enzyme-substrate reactions known in the art can be used herein.

[00206] Very small particles, termed nanoparticles, also can be used as detectable labels or detectable molecules to label decoder probes or any nucleic acids. These particles range from 1-1000 nm in size and include diverse chemical structures such as gold and silver particles and quantum dots.

[00207] When irradiated with angled incident white light, silver or gold nanoparticles ranging from 40-120 nm will scatter monochromatic light with high intensity. The wavelength of the scattered light is dependent on the size of the particle. Four to five different particles in close proximity will each scatter monochromatic light which when superimposed will give a specific, unique color. Alternatively, the gold nanoparticles can be detected or “developed” using a silver-based developer such that the tiny nanoparticle can be detected easily, even with naked eyes. The particles are being manufactured by companies such as Genicon Sciences. Derivatized silver or gold particles can be generally attached to a broad array of molecular molecules including, proteins, antibodies, small molecules, receptor ligands, and nucleic acids. For example, the surface of the particle can be chemically derivatized to allow attachment to a nucleotide, which can then be incorporated into a decoder probe.

[00208] Another type of nanoparticle that can be used as a detectable molecule or detectable label are quantum dots. Quantum dots are fluorescing crystals 1-5 nm in diameter that are excitable by a large range of wavelengths of light. These crystals emit light, such as monochromatic light, with a wavelength dependent on their chemical composition and size. Quantum dots such as CdSe, ZnSe, InP, or InAs possess unique optical properties.

[00209] Due to their very small size the quantum dots can be generally coupled into oligonucleotides directly without affecting the solubility or use of the oligonucleotide. Thus, quantum dots can be coupled to decoder probes as described herein. To synthesize a decoder probe-quantum dot complex by conventional batch chemistry, both the decoder probe and the

quantum dot require at least a reactive group of different kinds that can be reacted with each other. For example, if a decoder probe has an amino group and a quantum dot has an aldehyde group, these groups can react to form a Schiff base. A decoder probe can be derivatized to attach a single amino or other functional group using chemistry well known in the art. When a quantum dot is derivatized, the quantum dot can be covered with a chemical reagent which results in coating the entire surface of the nanoparticle with several functional groups.

[00210] A detectable molecule or detectable label can be attached to a nucleotide of a decoder probe or nucleic acid using a variety of methods well known in the art and described herein. For example, the detectable molecule or detectable label can be directly attached to the nucleotide in a 1:1 correspondence by incorporation of a radioactive phosphate into the phosphate backbone of the nucleotide. Also, for example, a general method for labeling phosphates with a fluorescent label that employs an imidazole derivative prepared from a BODIPY FL hydrazide has been reported (Wang and Giese, *Anal. Chem.* 65: 3518 (1993)).

[00211] Depending on the labeling moiety used, it can be desirable to derivatize or chemically modify a nucleotide in order to bind the label monomer. These methods and chemistries are known in the art. In addition, a linker can be used to attach a detectable molecule or detectable label to a nucleotide in a 1:1 correspondence. For example, a fluorescently labeled nucleotide such as fluorescein-12-dUTP can have a fluorophore monomer attached via a four-atom aminoalkynyl group to the dUTP molecule.

[00212] These nucleotides attached to detectable molecules or detectable labels can be incorporated into a decoder probe or nucleic acid using several methods for labeling nucleic acids well known in the art. For example, enzymes such as DNA or RNA polymerases, Taq polymerases, terminal deoxynucleotidyl transferases, or reverse transcriptases can be used to incorporate labeled nucleotides into decoder probes or nucleic acids.

[00213] Labeled nucleotides can be incorporated into decoder probe sequences or nucleic acids, for example, by nick translation. In this procedure DNase I is used to create single-strand nicks in double stranded DNA and then the 5' to 3' exonuclease and 5' to 3' polymerase actions of *E. coli* DNA polymerase I are used to remove stretches of single stranded DNA starting at the nicks and replace them with new strands made by incorporation of labeled nucleotides. Nick translation can utilize any labeled nucleotide including radioactively labeled nucleotides and biotinylated or digoxigenin labeled nucleotides. In a similar way T4 DNA polymerase can be

used to incorporate labeled nucleotides. In addition, labeled nucleotides can be incorporated into nucleic acids using the polymerase chain reaction (PCR) and Taq polymerases. The degree of labeling can be controlled by including one, or up to all four labeled nucleotides. In addition, the degree of labeling can be controlled by increasing or decreasing the concentration of the labeled nucleotide(s).

[00214] Other methods for labeling decoder probes or nucleic acids include generating single-stranded cDNA from RNA by using a reverse transcriptase in the presence of labeled nucleotides. In addition, DNA can be cloned into a vector with SP6 or T7 polymerase sites. Transcription in the presence of SP6 or T7 RNA polymerase and labeled nucleotides results in a labeled RNA transcript. The transcript can be labeled to different degrees by including one or more labeled nucleotides. In addition, several nucleotides within a nucleic acid can be labeled, for example, by cloning DNA into a bacteriophage M13 based vector. Then the Klenow fragment of DNA polymerase I and the M13 universal probe primer can be used to synthesize the complementary stand with incorporation of labeled nucleotides.

[00215] Several methods are described above for incorporation of labeled nucleotides into newly synthesized decoder probes or nucleic acids. Existing nucleic acids can also be labeled using several methods known in the art. For example, RNA or DNA can be end-labeled with [γ -³²P]ATP and T4 polynucleotide kinase. This kinase can be used to transfer the radioactive phosphate of ATP to a free 5' OH group in either DNA or RNA. The enzyme also has a phosphatase activity and so two reactions are possible. In the forward reaction, the enzyme catalyzes phosphorylation following removal of 5' terminal phosphates with alkaline phosphatase (or other phosphatase). In the exchange reaction, the kinase catalyzes the exchange of an existing 5' phosphate with the third or γ phosphate of ATP. The latter reaction is carried out in the presence of excess ATP and ADP for efficient phosphorylation. Using this method the radioactive phosphate of ATP is transferred to the end of the nucleic acid molecule.

[00216] Decoder probes or nucleic acids can also be labeled with terminal deoxynucleotidyl transferase which adds labeled nucleotides onto the 3' end of DNA fragments. Both single and double-stranded DNAs are substrates for this enzyme. The large (Klenow) fragment of *E. coli* DNA polymerase I can also be used to label the ends of decoder probes or nucleic acids. Since this enzyme has a 5' to 3' polymerase activity it can be used to "fill in" the 3' ends of nucleic acid (e.g., DNA) fragments opposite of 5' extensions or overhangs with labeled nucleotides. End-

labeling of decoder probes or nucleic acids using polynucleotide kinase or terminal deoxynucleotidyl transferase results in the incorporation of one detectable label or detectable molecule per nucleic acid. The “fill in” reaction can be used to label the decoder probe or nucleic acid at one nucleotide per nucleic acid or at more than one nucleotide per nucleic acid.

[00217] In addition, decoder probes or nucleic acids can be labeled by modification of nucleotides within the nucleic acid sequences. For example, cytidine residues in DNA and RNA can be modified by reaction with sodium bisulfite to form sulfonate intermediates that can then be directly coupled to hydrazides or aliphatic amines. Virtually any of the fluorescent, biotin or other hydrazides or aliphatic amines can be used in this reaction. The bisulfite-activated cytidylic acid can also be coupled to aliphatic diamines such as ethylenediamine. The amine-modified nucleic acids (e.g., DNA or RNA) can then be modified with any of the amine-reactive dyes. In addition, phosphate groups can be targeted in nucleic acids for labeling. Although phosphate groups of nucleotides are not very reactive in aqueous solution, their terminal phosphate groups can react with carbodiimides and similar reagents in combination with nucleophiles to yield labeled phosphodiester, phosphoramidate and phosphorothioate. For example, nucleic acids (e.g., DNA) can be reacted quantitatively with carbonyl diimidazole and a diamine such as ethylenediamine to yield a phosphoramidate that has a free primary amine and that this amine can then be modified with amino-reactive reagents. Fluorescent or biotinylated amines have been coupled to the 5' phosphate of tRNA using dithiodipyridine and triphenylphosphine.

[00218] The bond between detectable molecules or detectable labels and decoder probes or nucleic acids can be covalent bonds or non-covalent bonds that are stable to hybridization. The detectable molecules or detectable labels can be bound to a decoder probe or a nucleic acid in a sequence specific manner, for example by the incorporation of a labeled nucleotide into a sequence that has been digested by a restriction enzyme. Alternatively the detectable molecules or detectable labels can be bound to a decoder probe or a nucleic acid in a non-sequence specific manner, for example by the incorporation of a label onto the terminal phosphate of a nucleic acid using [γ -³²P]ATP and T4 polynucleotide kinase.

Synthesis of nucleic acids (e.g., for at least part of the nucleic acid label such as pre-determined subsequences and/or decoder probe sequences)

[00219] The nucleic acids described herein can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the label attachment region and the annealed complementary polynucleotide sequences or segments, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the synthetic nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl)uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N-6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N2-carboxypropyl)uracil, (acp3)w, and 2,6-diaminopurine.

[00220] Alternatively, the synthetic nucleic acid can be produced biologically using a vector into which a nucleic acid has been subcloned. As one example, a linear single-stranded DNA backbone can be made from a double stranded plasmid DNA using a four step protocol that includes (i) linearization of the dsDNA with a restriction enzyme, (ii) dephosphorylation with a thermolabile phosphatase, (iii) digestion with a second restriction enzyme to separate the cloning vector from the backbone sequence, and (iv) digestion with a strand-specific lambda exonuclease digestion, leaving only one strand of the backbone fragment intact.

[00221] In various embodiments, the nucleic acids can be modified at the base moiety, sugar moiety or phosphate backbone to improve, e.g., the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acids can be modified to generate peptide nucleic acids (see Hyrup et al, 1996, Bioorganic & Medicinal Chemistry 40:5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, e.g., DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral

backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup et al, 1996, *Bioorganic & Medicinal Chemistry* 4(1): 5-23; Perry-O'Keefe et al., 1996, *Proc. Natl. Acad. Sci. USA* 93: 14670-675.

[00222] In an exemplary embodiment, the selected novel nucleic acid sequence (e.g., DNA sequence) can be constructed synthetically as double-stranded nucleic acid by a commercial gene synthesis company and cloned in an oriented fashion into a “phagemid”, a plasmid vector containing an M13 or f1 phage intergenic (IG) region which contains the cis-acting sequences necessary for nucleic acid (e.g., DNA) replication and phage encapsidation, such as pUC119. The appropriate orientation of the cloned insert relative to the phage origin of replication allows for the generation of a single-stranded DNA backbone which is the reverse complement of the RNA molecules generated by in vitro transcription for each label attachment region.

[00223] In order to generate the single-stranded nucleic acid (e.g., DNA) backbone (e.g., of at least part of the nucleic acid label and/or decoder probes), the phagemid is transformed into an *E. coli* strain containing an F' episome. Subsequent infection of the transformed bacteria with a helper phage such as the M13 mutant K07 results in the secretion of the phagemid carrying the single-stranded nucleic acid sequence, packaged phage from which the circular, single-stranded nucleic acid is prepared using a standard protocol. This nucleic acid is linearized and the vector portion is excised by annealing short, complementary oligonucleotides to either end of the single-strander nucleic acid sequence to generate double-stranded restriction sites, followed by treatment with the appropriate restriction enzymes.

Analytes or target molecules

[00224] The terms “analyte,” “target analyte,” and “target molecule”, as used interchangeably herein, refer to the molecule detected, identified or measured by binding of a detection reagent described herein whose probe reagent(s) recognize (i.e., are specific binding partners) thereto. In some embodiments, a target molecule or an analyte can be, but is not limited to, any of the following or any combinations of the following: nucleic acid, peptide, a polypeptide/protein (e.g., a bacterial or viral protein or an antibody), a lipid, a carbohydrate, a glycoprotein, a glycolipid, a small molecule, an organic monomer, sugar, peptidoglycan, a cell, a virus or a drug.

Nucleic acids that can be analyzed by the methods herein include: double-stranded DNA, single-stranded DNA, single-stranded DNA hairpins, DNA/RNA hybrids, RNA (e.g. mRNA or miRNA) and RNA hairpins. Generally, a target molecule can be a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA. In other embodiments, a target molecule can be modified, e.g., by mutation or chemical reaction. In some embodiments, a target molecule can be synthetic or recombinant.

[00225] In some embodiments, an analyte can be an analyte comprising at least one post-translational modification, e.g., phosphorylations and/or glycosylations.

[00226] A target molecule or an analyte can be part of a sample that contains other components or can be the sole or major component of the sample. A target molecule or an analyte can be a component of a whole cell, tissue or body fluid, a cell or tissue extract, a fractionated lysate thereof or a substantially purified molecule. The target molecule can be present in solution or attached to a solid substrate, including, for example, to a solid surface such as a chip, microarray, bead or a blotting membrane. Also the target molecule or analyte can have either a known or unknown structure or sequence.

Sample

[00227] The methods, detection reagents and kits described herein can be used to analyze a sample from any sources, e.g., but not limited to biological samples (e.g., collected from organisms, animals or subjects), environmental samples, food, food byproduct, soil, archaeological samples, extraterrestrial samples, or any combinations thereof.

[00228] In some embodiments, the term “sample” refers to a biological sample. The term “biological sample” as used herein denotes a sample taken or isolated from a biological organism, e.g., tissue cell culture supernatant, cell lysate, a tissue sample (e.g., biopsy), a homogenate of a tissue sample from a subject or a fluid sample from a subject. Exemplary biological samples include, but are not limited to, blood, sputum, urine, cerebrospinal fluid, urine, sweat, mucus, nasal discharge, vaginal fluids, spinal fluid, pleural fluid, nipple aspirates, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, feces, sperm, cells or cell cultures, serum, leukocyte fractions, smears, tissue samples of all kinds, plants and parts of plants, microorganisms (such as bacteria), viruses (such as cytomegalo virus, HIV, hepatitis B, hepatitis C, hepatitis δ virus), yeasts, embryos, fungi, cell-

free sample material, etc. The term also includes both a mixture of the above-mentioned samples such as fungus-infected plants or whole human blood containing mycobacteria as well as food samples that contain free or bound nucleic acids, or proteins, or cells containing nucleic acids or proteins, environmental samples which contain free or bound nucleic acids, or proteins, or cells containing nucleic acids or proteins. The term “biological sample” also includes untreated or pretreated (or pre-processed) biological samples.

[00229] A “biological sample” can contain cells from subject, but the term can also refer to non-cellular biological material, such as non-cellular fractions of blood, saliva, or urine, that can be used to measure gene expression levels. In some embodiments, the sample is from a resection, bronchoscopic biopsy, or core needle biopsy of a primary or metastatic tumor, or a cell block from pleural fluid. In addition, fine needle aspirate samples can be used. Samples can be either paraffin-embedded or frozen tissue. In some embodiments, a biological sample can comprise a biopsy, a surgically removed tissue, a swap, or any combinations thereof.

[00230] The sample can be obtained by removing a sample of cells from a subject, but can also be accomplished by using previously isolated cells (e.g. isolated by another person). In addition, the biological sample can be freshly collected or a previously collected sample. Furthermore, the biological sample can be utilized for the detection of the presence and/or quantitative level of a biomolecule of interest. Representative target analytes include, but are not limited to nucleic acids, proteins, and derivatives and fragments thereof.

[00231] In some embodiments, the biological sample is an untreated biological sample. As used herein, the phrase “untreated biological sample” refers to a biological sample that has not had any prior sample pre-treatment except for dilution and/or suspension in a solution. Exemplary methods for treating a biological sample include, but are not limited to, centrifugation, filtration, sonication, homogenization, heating, freezing and thawing, and any combinations thereof.

[00232] In accordance with some embodiments of various aspects described herein, a biological sample can be pre-processed, as described earlier, before employing the detection reagents and the methods described herein. In some embodiments, the biological sample can be filtered before isolating a cellular material according to the methods, apparatus and kits described herein.

[00233] In some embodiments, the biological sample can be treated with a chemical and/or biological reagent. Chemical and/or biological reagents can be employed to protect and/or maintain the stability of the sample or target analytes during processing. In addition, or alternatively, chemical and/or biological reagents can be employed to release or expose target analytes from other components of the sample. One exemplary reagent is a protease inhibitor, which is generally used to protect or maintain the stability of the target analytes during processing.

[00234] In some embodiments, the term “sample” as used herein can refer to an environmental sample (including, but not limited to, air, agricultural (e.g., but not limited to hydrofarms or hydroponic samples), pond, water, wastewater, and soil samples); biological warfare agent samples; research samples including extracellular fluids. In some embodiments, an environmental sample can comprise a sample collected from a working surface of an equipment or machine (e.g., but not limited to, food or pharmaceutical product processing equipment or machine), a device (e.g., but not limited to, biomedical devices, implantation devices, fluid delivery devices such as a tubing, and/or a catheter), and/or a building or dwellings (e.g., but not limited to, food processing plants, pharmaceutical manufacturing plants, hospitals, and/or clinics).

[00235] In some embodiments, a sample can comprise food (e.g., solid and/or fluid food as well as processed food) and/or food byproduct. For example, the methods, detection reagents and kits described herein can be used to detect an analyte, e.g., a particular nutrient, in food and/or food byproduct, e.g., but not limited to, meat, milk, yoghurt, bread, starch-based products, vegetables, and any combinations thereof. In some embodiments, the methods, detection reagents and kits described herein can be used to detect a contaminant, e.g., bacteria, fungus, spores, molds, and/or viruses, in food and/or food byproduct.

[00236] In some embodiments, a sample can comprise a pharmaceutical product (e.g., but not limited to pills, tablets, gel capsules, syrups, vaccines, liquids, sprays, and any combinations thereof). For example, the methods, detection reagents and kits described herein can be used to detect the presence and/or measure the level of a particular active agent present in a pharmaceutical product. In some embodiments, the methods, detection reagents and kits described herein can be used to detect a contaminant, e.g., bacteria, fungus, spores, molds, and/or viruses, in a pharmaceutical product.

[00237] In some embodiments, a sample can comprise an archaeological sample. In some embodiments, an archaeological sample can be obtained or collected from artifacts, architecture, biofacts (or ecofact, e.g., an object found at an archaeological site), cultural landscapes, and any combinations thereof.

[00238] In some embodiments, a sample can comprise an extraterrestrial sample. For example, an extraterrestrial sample can be any object or specimen (e.g., rock, meteorite, and/or environmental samples) obtained or collected from outer space or universe, and/or planets beyond the planet Earth, e.g., the moon, other planets (e.g., but not limited to, Mars, and/or Jupiter) and/or non-stellar objects.

Applications of the detection reagents

[00239] The detection reagents, compositions, methods and kits described herein can be used for diagnostic, prognostic therapeutic and screening purposes. The inventions described herein provide the advantage that many different target analytes can be analyzed at one time from a single sample using the methods described herein. This allows, for example, for several diagnostic tests to be performed on one sample.

[00240] When the probe reagent is a nucleic acid, the methods described herein can discriminate between nucleotide sequences. The difference between the target nucleotide sequences can be, for example, a single nucleic acid base difference, a nucleic acid deletion, a nucleic acid insertion, or rearrangement. Such sequence differences involving more than one base can also be detected. In some embodiments, the oligonucleotide probe sets have substantially the same length so that they hybridize to target nucleotide sequences at substantially similar hybridization conditions. As a result, the process described herein is able to detect various kinds of diseases or disorders, e.g., but not limited to, infectious diseases, genetic diseases, and cancer.

[00241] Without wishing to be bound, the detection reagents, compositions, methods and kits described herein can also be used for environmental monitoring, forensics, and food science. Examples of genetic analyses that can be performed on nucleic acids include e.g., SNP detection, STR detection, RNA expression analysis, promoter methylation, gene expression, virus detection, viral subtyping and drug resistance.

[00242] In the area of environmental monitoring, some embodiments of various aspects described herein can be used for detection, identification, and monitoring of pathogenic and indigenous microorganisms in natural and engineered ecosystems and microcosms such as in municipal waste water purification systems and water reservoirs or in polluted areas undergoing bioremediation. It can also be used to detect plasmids containing genes that can metabolize xenobiotics, to monitor specific target microorganisms in population dynamic studies, or either to detect, identify, or monitor genetically modified microorganisms in the environment and in industrial plants.

[00243] Some embodiments of various aspects described herein can also be used in a variety of forensic areas, including for human identification for military personnel and criminal investigation, paternity testing and family relation analysis, HLA compatibility typing, and screening blood, sperm, or transplantation organs for contamination.

[00244] In the food and feed industry, some embodiments of various aspects described herein have a wide variety of applications. For example, it can be used for identification and characterization of production organisms such as yeast for production of beer, wine, cheese, yogurt, bread, etc. Another area of use is related to the quality control and certification of products and processes (e.g., livestock, pasteurization, and meat processing) for contaminants. Other uses include the characterization of plants, bulbs, and seeds for breeding purposes, identification of the presence of plant-specific pathogens, and detection and identification of veterinary infections.

[00245] In some embodiments, the methods, detection reagents and kits described herein can be used to detect the presence and/or measure the level of a particular active agent present in a pharmaceutical product. In some embodiments, the methods, detection reagents and kits described herein can be used to detect a contaminant, e.g., bacteria, fungus, spores, molds, and/or viruses, in a pharmaceutical product.

[00246] In some embodiments, the methods, detection reagents and kits described herein can be used to detect the presence and/or measure the level of an analyte of interest present in an archaeological sample, e.g., obtained or collected from artifacts, architecture, biofacts (or ecofact, e.g., an object found at an archaeological site), cultural landscapes, and any combinations thereof.

[00247] In some embodiments, the methods, detection reagents and kits described herein can be used to detect the presence and/or measure the level of an analyte of interest present in an extraterrestrial sample, e.g., any object or specimen (e.g., rock, meteorite, and/or environmental samples) obtained or collected from outer space or universe, and/or planets beyond the planet Earth, e.g., the moon, other planets (e.g., but not limited to, Mars, and/or Jupiter) and/or non-stellar objects. For example, the methods, detection reagents, and kits described herein can be used to analyze the composition of an extraterrestrial sample or specimen, e.g., a rock specimen, a water sample, and/or an air sample.

[00248] In some embodiments, the detection reagents and methods described herein can be used to detect and/or identify analytes comprising a post-translation modification, e.g., phosphorylation or glycosylation. As such, in some embodiments, the detection reagents and methods described herein can differentiate between multiple different glycosylation forms of recombinant therapeutic proteins

[00249] ***Immunohistochemistry:*** Some embodiments of various aspects described herein can be used to perform antibody-based staining of cell or tissues for microscopic evaluation. This can involve either unfixed or unpermeabilized samples examined for surface or extracellular antigens, or permeabilized samples wherein intracellular antigens are also probed. While very common, immunohistochemistry is typically limited to probing with a small set of antibodies at a time (due to the limitation of available optical colors), and multiple staining cycles are generally avoided, since the stripping of the preceding cycle's antibodies can damage the sample. Some embodiments of the detection reagents comprising antibodies as probe reagents and nucleic acid labels can allow for many antibodies to be used concurrently, which saves time and sample material, extract more data, and demand less prior knowledge of the sample. In some embodiments, without wishing to be bound by theory, when a subset of the probed antigens can overlap spatially, the detection reagents with a plurality of contiguous pre-determined subsequences (e.g., each pre-determined subsequences contain one nucleotide, as shown in **Fig. 1**) can be used.

[00250] ***In-situ hybridization:*** Fluorescence in-situ hybridization (FISH) is a technique for detecting (and/or quantifying) the presence of certain cellular DNA or RNA (often ribosomal RNA). As with other fluorescence-based techniques, FISH is limited to the number of colors available to the microscopy. Using some embodiments of the detection reagents and/or methods

described herein, the cells can be probed for many sequences simultaneously, thus allowing the user to save time and sample material, extract more data, and demand less prior knowledge of the sample.

[00251] *Expression profiling:* Nucleic-acid probe reagents of the detection reagents can target messenger RNA or micro RNA and provide information on the RNA-level expression state of the cell. Using some embodiments of the detection reagents and/or methods described herein, the assay can capture and probe numerous mRNA and/or miRNA at once, reducing and/or eliminating potentially harmful stripping steps.

[00252] *Western blots:* Western blots are protein assays wherein proteins are separated electrophoretically, transferred to a membrane and stained. In many cases, the staining is done using one or a few antibodies in order to detect the corresponding antigens in the blot. By using some embodiments of the detection reagents and/or methods described herein, the blot can be simultaneously probed using a large number of antibodies without antibody-stripping steps. This can allow one blot to provide significantly more information than the conventional Western blots where one antibody is usually probed at a time and can require antibody stripping for the next antibody probing, thereby saving sample material and time.

[00253] *Diagnostic/Prognostic Methods:* The present methods can be applied to the analysis of biological samples obtained or derived from a patient so as to determine whether a diseased cell type is present in the sample and/or to stage the disease.

[00254] In some embodiments, the methods described herein are used in the diagnosis of a condition. As used herein the term “diagnose” or “diagnosis” of a condition includes predicting or diagnosing the condition, determining predisposition to the condition, monitoring treatment of the condition, diagnosing a therapeutic response of the disease, and prognosis of the condition, condition progression, and response to particular treatment of the condition. For example, a blood sample can be assayed according to any of the methods described herein to determine the presence and/or quantity of markers of a cancerous cell type in the sample, thereby diagnosing or staging the cancer.

[00255] In some embodiments, the detection reagents and methods described herein can be directly used as contrast agents for in vivo or in situ diagnosis, in which detection reagents are administered to a subject, either by oral administration or local injection. The probe reagents of the detection reagents can bind to the target analytes, e.g., biomarkers for specific diseases or

disorders, and detection of the nucleic acid labels using the methods described herein can then locate the target analytes. In some embodiments, the detection reagents and methods described herein can be used to locate microscopic tumors in a subject, where other conventional methods are not sensitive enough to detect such microscopic tumors.

[00256] Cancers which can be detected by some embodiments of the process described herein generally involve oncogenes, tumor suppressor genes, or genes involved in DNA amplification, replication, recombination, or repair. Examples of these include: BRCA1 gene, p53 gene, APC gene, Her2/Neu amplification, Bcr/Ab1, K-ras gene, and human papillomavirus Types 16 and 18. Some embodiments of various aspects can be used to identify amplifications, large deletions as well as point mutations and small deletions/insertions of the above genes in the following common human cancers: leukemia, colon cancer, breast cancer, lung cancer, prostate cancer, brain tumors, central nervous system tumors, bladder tumors, melanomas, liver cancer, osteosarcoma and other bone cancers, testicular and ovarian carcinomas, head and neck tumors, and cervical neoplasms.

[00257] Genetic diseases can also be detected by some embodiments of the process described herein. This can be carried out by prenatal or post-natal screening for chromosomal and genetic aberrations or for genetic diseases. Examples of detectable genetic diseases include: 21 hydroxylase deficiency, cystic fibrosis, Fragile X Syndrome, Turner Syndrome, Duchenne Muscular Dystrophy, Down Syndrome or other trisomies, heart disease, single gene diseases, HLA typing, phenylketonuria, sickle cell anemia, Tay-Sachs Disease, thalassemia, Klinefelter Syndrome, Huntington Disease, autoimmune diseases, lipidosis, obesity defects, hemophilia, inborn errors of metabolism, and diabetes.

[00258] Alternatively, the methods described herein can be used to diagnose pathogen infections, for example infections by intracellular bacteria and viruses, by determining the presence and/or quantity of markers of bacterium or virus, respectively, in the sample.

[00259] A wide variety of infectious diseases can be detected by some embodiments of the process described herein. Typically, these are caused by bacterial, viral, parasite, and fungal infectious agents. The resistance of various infectious agents to drugs can also be determined using some embodiments of various aspects described herein.

[00260] Bacterial infectious agents which can be detected by some embodiments of various aspects described herein can include *Escherichia coli*, *Salmonella*, *Shigella*, *Klebsiella*,

Pseudomonas, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Mycobacterium aviumintracellulare*, *Yersinia*, *Francisella*, *Pasteurella*, *Brucella*, *Clostridia*, *Bordetella pertussis*, *Bacteroides*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, B-Hemolytic strep., *Corynebacteria*, *Legionella*, *Mycoplasma*, *Ureaplasma*, *Chlamydia*, *Neisseria gonorrhea*, *Neisseria meningitidis*, *Hemophilus influenzae*, *Enterococcus faecalis*, *Proteus vulgaris*, *Proteus mirabilis*, *Helicobacter pylori*, *Treponema palladium*, *Borrelia burgdorferi*, *Borrelia recurrentis*, Rickettsial pathogens, *Nocardia*, and *Acetivomycetes*.

[00261] Fungal infectious agents which can be detected by some embodiments of various aspects described herein can include *Cryptococcus neoformans*, *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Paracoccidioides brasiliensis*, *Candida albicans*, *Aspergillus fumigatus*, *Phycomycetes (Rhizopus)*, *Sporothrix schenckii*, *Chromomycosis*, and *Maduromycosis*.

[00262] Viral infectious agents which can be detected by some embodiments of various aspects described herein can include human immunodeficiency virus, human T-cell lymphocytotropic virus, hepatitis viruses (e.g., Hepatitis B Virus and Hepatitis C Virus), Epstein-Barr Virus, cytomegalovirus, human papillomaviruses, orthomyxo viruses, paramyxo viruses, adenoviruses, corona viruses, rhabdo viruses, polio viruses, toga viruses, bunya viruses, arena viruses, rubella viruses, and reo viruses.

[00263] Parasitic agents which can be detected by some embodiments of various aspects described herein can include *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium vivax*, *Plasmodium ovale*, *Onchocerca volvulus*, *Leishmania*, *Trypanosoma* spp., *Schistosoma* spp., *Entamoeba histolytica*, *Cryptosporidium*, *Giardia* spp., *Trichomonas* spp., *Balantidium coli*, *Wuchereria bancrofti*, *Toxoplasma* spp., *Enterobius vermicularis*, *Ascaris lumbricoides*, *Trichuris trichiura*, *Dracunculus medinensis*, trematodes, *Diphyllobothrium latum*, *Taenia* spp., *Pneumocystis carinii*, and *Necator americanus*.

[00264] In some embodiments, contacting a biological sample with the detection reagents described herein immune-staining, in-situ hybridization or a combination, can be used for the purpose of detecting and identifying pathogens in biological samples. In such embodiments, for example, surface or intracellular antigens, DNA, ribosomal RNA and/or messenger RNA can be detected with some embodiments of the detection reagents and methods described herein. Detection reagents and methods described herein can be especially useful in this context since a)

which pathogen may be in the sample is not known in advance, and b) each pathogen cell should respond to one or few of the probes, facilitating the detection reagent and nucleic acid label design.

[00265] Some embodiments of various aspects described herein can also be used for detection of drug resistance by infectious agents. For example, vancomycin-resistant *Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae*, multi-drug resistant *Mycobacterium tuberculosis*, and AZT-resistant human immunodeficiency virus can all be identified with some embodiments of various aspects described herein.

[00266] Thus, the target molecules detected using the compositions and methods described herein can be either patient markers (such as a cancer marker) or markers of infection with a foreign agent, such as bacterial or viral markers.

[00267] Because of the quantitative nature of detection reagents, the compositions and methods described herein can be used to quantitate target molecules whose abundance is indicative of a biological state or disease condition, for example, blood markers that are upregulated or downregulated as a result of a disease state.

[00268] In addition, the compositions and methods described herein can be used to provide prognostic information that assists in determining a course of treatment for a patient. For example, the amount of a particular marker for a tumor can be accurately quantified from even a small sample from a patient. For certain diseases like breast cancer, overexpression of certain genes, such as Her2-neu, indicate a more aggressive course of treatment will be needed.

[00269] In some embodiments, the compositions and methods described herein can be administered to a subject for in vivo diagnosis and/or monitoring of a disease or disorder. Specific devices or methods known in the art for the in vivo detection of fluorescence, e.g., from fluorophores or fluorescent proteins, include, but are not limited to, in vivo near-infrared fluorescence (see, e.g., Frangioni, *Curr. Opin. Chem. Biol.*, 7:626-634 (2003)), the MaestroTM in vivo fluorescence imaging system (Cambridge Research & Instrumentation, Inc.; Woburn, Mass.), in vivo fluorescence imaging using a flying-spot scanner (see, e.g., Ramanujam et al, *IEEE Transactions on Biomedical Engineering*, 48:1034-1041 (2001), and the like. Other methods or devices for detecting an optical response include, without limitation, visual inspection, CCD cameras, video cameras, photographic film, laser-scanning devices,

fluorometers, photodiodes, quantum counters, epifluorescence microscopes, scanning microscopes, flow cytometers, fluorescence microplate readers, or signal amplification using photomultiplier tubes.

[00270] Any device or method known in the art for detecting the radioactive emissions of radionuclides in a subject is suitable for use in various aspects described herein. For example, methods such as Single Photon Emission Computerized Tomography (SPECT), which detects the radiation from a single photon gamma-emitting radionuclide using a rotating gamma camera, and radionuclide scintigraphy, which obtains an image or series of sequential images of the distribution of a radionuclide in tissues, organs, or body systems using a scintillation gamma camera, may be used for detecting the radiation emitted from a radiolabeled aggregate. Positron emission tomography (PET) is another suitable technique for detecting radiation in a subject.

[00271] *Analysis of Pathology Samples:* RNA extracted from formaldehyde- or paraformaldehyde-fixed paraffin-embedded tissue samples is typically poor in quality (fragmented) and low in yield. This makes gene expression analysis of low-expressing genes in histology samples or archival pathology tissues extremely difficult and often completely infeasible. The detection reagents and methods described herein can fill this unmet need by allowing the analysis of very small quantities of low-quality total RNA.

[00272] To use detection reagents in such an application, total RNA can be extracted from formaldehyde- or paraformaldehyde-fixed paraffin-embedded tissue samples (or similar) using commercially available kits such as RecoverAll Total Nucleic Acid Isolation Kit (Ambion) following manufacturer's protocols. RNA in such samples is frequently degraded to small fragments (200 to 500 nucleotides in length), and many paraffin-embedded histology samples only yield tens of nanograms of total RNA. Small amounts (5 to 100 ng) of this fragmented total RNA can be used directly as target analyte upon contact with the detection reagents described herein, using the methods described herein described herein.

[00273] *Screening Methods:* The methods described herein can be used, among other things, for determining the effect of a perturbation, including chemical compounds, mutations, temperature changes, growth hormones, growth factors, disease, or a change in culture conditions, on various target molecules, thereby identifying target molecules whose presence, absence or levels are indicative of particular biological states. In one embodiment, some aspects described herein can be used to elucidate and discover components and pathways of disease

states. For example, the comparison of quantities of target molecules present in a disease tissue with “normal” tissue allows the elucidation of important target molecules involved in the disease, thereby identifying targets for the discovery/screening of new drug candidates that can be used to treat disease.

[00274] *Targeted Delivery Vehicles:* In some embodiments, the therapeutic and/or diagnostic agent can be encapsulated within the particles that provide conjugation sites for the probe reagents and nucleic acid labels. In such embodiments, the detection reagents and methods described herein can be used to deliver a therapeutic and/or diagnostic agent to cells where the probe reagents of the detection reagents bind. Nucleic acid labels of the detection reagents can be detected to monitor the location of drug administration.

[00275] *Cell Lineage Tracking:* In some embodiments, the detection reagents described herein can be used to track cell lineage, e.g., in culture or in vivo. For example, the probe reagents of the detection reagents can bind to live cells of interest, allowing labeling and/or tracking of individual cell differentiation. Examples of such application include, but are not limited to, lineage tracking in stem cell differentiation, and lineage determination of dendritic cells and/or white blood cells. In some embodiments, these cells after lineage determination can be further used accordingly.

Kits comprising detection reagents

[00276] Kits for various assays are also provided herein. In some embodiments, a kit can comprise: (a) a plurality of nucleic acid labels of the detection reagents described herein; and (b) at least one coupling reagent required to conjugate the nucleic acid labels to probe reagents of interest. In such embodiments, users can attach the provided nucleic acid labels to their probe reagents of interest to form their own detection reagents described herein. Examples of the coupling reagent required for nucleic acid label-probe reagent conjugation can include any reagents that are generally used to perform any of the conjugation methods described herein, e.g., biotin and avidin-like molecules such as streptavidin or neutravidin. However, in alternative embodiments, the kit can comprise a plurality of the detection reagents described herein that are ready for use and thus no nucleic acid label-probe reagent conjugation steps are required to be performed by users prior to use.

[00277] In some embodiments, the kit can further comprise at least one reagent, e.g., used in a readout method described herein. For example, if the readout method is sequencing-based, the kit can further comprise at least one agent used in sequencing-based readout. Alternatively, if the readout method is hybridization-based, the kit can further comprise at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.

[00278] Accordingly, in some embodiments, a kit for hybridization-based readout can comprise: (a) a plurality of nucleic acid labels of the detection reagents described herein; (b) at least one reagent required to conjugate the nucleic acid labels to probe reagents of interest; and (c) at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature. In some embodiments, the kit can further comprise at least one reagent, e.g., used in the readout method.

[00279] In alternative embodiments, a kit for hybridization-based readout can comprise: (a) a plurality of the detection reagents described herein; (b) at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature; and (c) at least one reagent.

[00280] In any embodiments of the kit described herein, the plurality of the detection reagents can include one or more different kinds of the detection reagents. In some embodiments, it is desirable to provide different kinds of the detection reagents (e.g., different types of probe reagents, target binding domains, and/or target analytes) in separate containers. The number of different kinds of the detection reagents provided in the kit can be tailored for each application described herein. In some embodiments, the kit can comprise at least 2, at least 3, at least 4, at least 5, at least 6 at least 7, at least 8, at least 9, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100 or more different kinds of the detection reagents.

[00281] In any embodiments of the kit described herein, the kit can comprise a plurality of sets of decoder probes, e.g., e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10 or more sets of decoder probes, wherein each set of decoder probes can target a different pre-determined sequence of the nucleic acid

label. In each set of the decoder probes, there can be about 2, 3, 4, 5, 6, 7, 8, 9, 10 or more distinct populations of decoder probes. The decoder probes can be pre-labeled with at least one detectable label or unlabeled. In some embodiments where the decoder probes are not pre-labeled, the kit can further comprise one or more distinct detectable labels provided in separate containers for labeling the decoder probes. In some embodiments, the detectable label can be an optical label described herein.

[00282] Examples of a reagent, e.g., used in a readout method, can include, but are not limited to, a readout reagent, a wash buffer, a signal removal buffer, buffers for performing hybridization reactions, restriction endonucleases, nucleic acid ligases, and any combinations thereof.

[00283] In some embodiments, the detection reagents can be provided in a solution phase. In other embodiments, the detection reagents can be immobilized in a solid support or substrate.

[00284] By “substrate” or “solid support” or other grammatical equivalents herein is meant any material that can be modified to contain discrete individual sites appropriate for the attachment or association of beads and is amenable to at least one detection method. As will be appreciated by those in the art, the number of possible substrates is very large. Possible substrates include, but are not limited to, glass and modified or functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, Teflon, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, optical fiber bundles, and a variety of other polymers. In general, the substrates allow optical detection and do not themselves appreciably fluoresce.

[00285] Generally the substrate is flat (planar), although as will be appreciated by those in the art, other configurations of substrates may be used as well; for example, three dimensional configurations can be used, for example by embedding the detection reagents in a porous block of plastic that allows sample access to the detection reagents and using a confocal microscope for detection. In some embodiments, the detection reagents can be placed on the inside surface of a tube, for flow-through sample analysis to minimize sample volume. Preferred substrates include optical fiber bundles, and flat planar substrates such as glass, polystyrene and other plastics and acrylics. In some embodiment, the solid support or substrate can be a multi-well plate.

[00286] In addition to the above mentioned components, the kit can include informational material. The informational material can be descriptive, instructional, marketing or other

material that relates to the methods described herein and/or the use of the aggregates for the methods described herein. For example, the informational material describes methods for administering the detection reagents to a subject, and/or includes instructions to label decoder probes with detectable labels provided therein and/or instructions to conjugate at least one nucleic acid label to a probe reagent. The kit can also include a delivery device.

[00287] In some embodiments, the informational material can include instructions to administer the formulation in a suitable manner, e.g., in a suitable dose, dosage form, or mode of administration (e.g., a dose, dosage form, or mode of administration described herein). In another embodiment, the informational material can include instructions for identifying a suitable subject, e.g., a human, e.g., an adult human. The informational material of the kits is not limited in its form. In many cases, the informational material, e.g., instructions, is provided in printed matter, e.g., a printed text, drawing, and/or photograph, e.g., a label or printed sheet. However, the informational material can also be provided in other formats, such as Braille, computer readable material, video recording, or audio recording. In another embodiment, the informational material of the kit is a link or contact information, e.g., a physical address, email address, hyperlink, website, or telephone number, where a user of the kit can obtain substantive information about the formulation and/or its use in the methods described herein. Of course, the informational material can also be provided in any combination of formats.

[00288] In some embodiments, the kit contains separate containers, dividers or compartments for each component and informational material. For example, each different component can be contained in a bottle, vial, or syringe, and the informational material can be contained in a plastic sleeve or packet. In other embodiments, the separate elements of the kit are contained within a single, undivided container. For example, the formulation is contained in a bottle, vial or syringe that has attached thereto the informational material in the form of a label.

[00289] In some embodiments, the kit includes a plurality, e.g., a pack, of individual containers, each containing one or more unit dosage forms of the composition comprising the detection reagents. For example, the kit includes a plurality of syringes, ampules, foil packets, or blister packs, each containing a single unit dose of the formulation. The containers of the kits can be air tight and/or waterproof.

[00290] *Embodiments of the various aspects described herein can be illustrated by the following numbered paragraphs.*

1. A method for detecting a plurality of analytes in a sample, comprising:
 - a. contacting the sample with a composition comprising a plurality of detection reagents, wherein each subpopulation of the detection reagents targets at least one different analyte, and wherein each detection reagent comprises:

at least one probe reagent targeting an analyte and at least one nucleic acid label comprising one or a plurality of pre-determined subsequences, wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together; and wherein at least a portion of said one or the plurality of pre-determined subsequences form an identifier of said at least one probe reagent; and
 - b. detecting in a temporally-sequential manner said one or the plurality of the pre-determined subsequences of said detection reagent, wherein said detection of the subsequences each generates a signal signature corresponding to said subsequence, and wherein a temporal order of the signal signatures corresponding to said one or the plurality of the subsequences of said detection reagent identifies a subpopulation of the detection reagents.
2. The method of paragraph 1, wherein said each subpopulation of the detection reagents targets a set of analytes (e.g., at least two analytes or more).
3. The method of paragraph 1 or 2, wherein the temporal order of the signal signatures corresponding to said one or the plurality of the subsequences of said detection reagent is unique for each subpopulation of the detection reagents.
4. The method of any of paragraphs 1-3, wherein said detection reagents are present in a soluble phase.
5. The method of any of paragraphs 1-4, further comprising processing said sample before said contacting with said plurality of detection reagents.
6. The method of any of paragraphs 1-5, further comprising removing any unbound detection reagents before the detecting step (b).
7. The method of any of paragraphs 1-6, further comprising comparing said temporal order of the signal signatures with different identifiers of said at least one probe reagent,

wherein an agreement between the temporal order of the signal signatures and a particular identifier of said at least one probe reagent identifies the analyte in the sample.

8. The method of any of paragraphs 1-7, further comprising measuring the intensity of the signal signatures generated from each subpopulation of the detection reagents.
9. The method of paragraph 8, wherein the intensity of the signal signatures generated from each subpopulation of the detection reagents indicates an amount of the analyte.
10. The method of paragraph 8 or 9, wherein the intensity of the signal signatures generated from each subpopulation of the detection reagents is used in identification of the subpopulation of the detection reagents.
11. The method of any of paragraphs 1-10, wherein said detecting of step (b) comprises sequencing.
12. The method of paragraph 11, wherein said sequencing is performed via ligation, hybridization, synthesis, amplification, or single-base extension.
13. The method of any of paragraphs 1-12 wherein said detecting of step (b) comprises hybridizing a decoder probe with said subsequence, wherein said decoder probe comprises a detectable label.
14. The method of any of paragraphs 1-13, wherein said detecting of step (b) comprises:
 - a. hybridizing a set of decoder probes with a subsequence of the detection reagents, wherein each subpopulation of the decoder probes comprises a detectable label, each detectable label producing a signal signature;
 - b. detecting said signal signature produced by the hybridization of said set of decoder probes;
 - c. optionally removing said different signal signature produced by the hybridization of said set of decoder probes; and
 - d. repeating steps (a) through (c) for other subsequences of said detection reagents, thereby producing a temporal order of the signal signatures corresponding to said each detection reagent.

15. The method of paragraph 14, wherein said each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.
16. The method of paragraph 14 or 15, wherein said each subpopulation of the decoder probes is at least partially or completely complementary to said subsequence of the detection reagents.
17. The method of any of paragraphs 14-16, wherein at least two or more subpopulations of the decoder probes are at least partially or completely complementary to the same subsequence of the detection reagents.
18. The method of any of paragraphs 14-17, wherein said removing step is performed by washing, heating, photobleaching, displacement, cleavage, enzymatic digestion, quenching, chemical degradation, bleaching, oxidation or any combinations thereof.
19. The method of any of paragraphs 14-18, wherein said detectable label comprises or is an optical label selected from the group consisting of a small-molecule dye, a fluorescent molecule, a fluorescent protein, a quantum dot, Raman label, a chromophore, and any combinations thereof.
20. The method of any of paragraphs 14-19, wherein said detectable label comprises or is a colorimetric reagent.
21. The method of any of paragraphs 14-20, wherein said detectable label comprises or is a Raman label.
22. The method of any of paragraphs 1-21, wherein said signal signatures comprise or are optical signatures.
23. The method of paragraph 22, wherein said optical signatures comprise signatures of fluorescent color, visible light, no-color, Raman label, or any combinations thereof.
24. The method of paragraph 22, wherein said optical signatures comprise signatures of one or more fluorescent colors, one or more visible lights, one or more no-colors, one or more Raman labels, or any combinations thereof.

25. The method of any of paragraphs 22-24, wherein said optical signatures are detected by optical imaging or spectroscopy.
26. The method of any of paragraphs 1-25, wherein said analytes are selected from the group consisting of antigens, receptors, proteins, peptides, sugars, glycoproteins, peptidoglycans, lipids, nucleic acids, oligonucleotides, cells, viruses, and any combinations thereof.
27. The method of any of paragraphs 1-26, wherein said nucleic acids are selected from the group consisting of cellular DNA or RNA, messenger RNA, microRNA, ribosomal RNA, and any combinations thereof.
28. The method of any of paragraphs 1-27, wherein said sample is a protein sample immobilized on a solid support.
29. The method of paragraph 28, wherein the solid support is a blotting membrane.
30. The method of any of paragraphs 1-27, wherein said sample is a biological sample.
31. The method of paragraph 30, wherein said biological sample comprises one or more cells, one or more tissues, one or more fluids or any combinations thereof.
32. The method of paragraph 30 or 31, wherein said biological sample comprises blood, sputum, cerebrospinal fluid, urine, saliva, sperm, sweat, mucus, nasal discharge, vaginal fluids or any combinations thereof.
33. The method of any of paragraphs 30-31, wherein the said biological sample comprises a biopsy, a surgically removed tissue, a swap or any combinations thereof.
34. The method of paragraphs 1-27, wherein said sample comprises an environmental sample, food, food byproduct, soil, an archaeological sample, an extraterrestrial sample, or any combinations thereof.
35. The method of any of paragraphs 1-34, wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together by at least one linker.
36. The method of paragraph 35, wherein said linker is a bond.
37. The method of paragraph 35 or 36, wherein said linker is a linker molecule.

38. The method of paragraph 37, wherein said linker molecule is a polymer, sugar, nucleic acid, peptide, protein, hydrocarbon, lipid, polyethylene glycol, crosslinker, or any combinations thereof.
39. The method of any of paragraphs 35-38, wherein said linker is a particle.
40. The method of paragraph 39, wherein said particle is selected from a group consisting of a gold nanoparticle, a magnetic bead or nanoparticle, a polystyrene bead, a nanotube, a nanowire, a microparticle, and any combinations thereof.
41. The method of paragraph 40, wherein said particle is a nanoparticle.
42. The method of any of paragraphs 39-41, wherein said particle is modified.
43. The method of any of paragraphs 39-42, wherein said particle is coated with streptavidin or a derivative thereof.
44. The method of any of paragraphs 39-43, wherein said particle is modified with at least one functional group.
45. The method of paragraph 44, wherein said at least one functional group is selected from the group consisting of amine, carboxyl, hydroxyl, aldehyde, ketone, tosyl, silanol, chlorine, hydrazine, hydrazide, photoreactive groups, and any combinations thereof.
46. The method of any of paragraphs 35-45, wherein said linker is multivalent.
47. The method of paragraph 46, wherein when the multivalent linker is an avidin-like molecule, both the probe reagent and the nucleic acid label are biotinylated.
48. The method of any of paragraphs 1-47, wherein said at least one probe reagent is selected from the group consisting of a nucleic acid, an antibody or a portion thereof, an antibody-like molecule, an enzyme, a cell, an antigen, a small molecule, a protein, a peptide, a peptidomimetic, a sugar, a carbohydrate, a lipid, a glycan, a glycoprotein, an aptamer, and any combinations thereof.
49. The method of any of paragraphs 1-48, wherein said at least one probe reagent is modified.
50. The method of any of paragraphs 1-49, wherein said at least one probe reagent is biotinylated.

51. The method of any of paragraphs 1-50, wherein said at least one nucleic acid label is single-stranded, double-stranded, partially double-stranded, a hairpin, linear, circular, branched, a concatemer, or any combinations thereof.
52. The method of any of paragraphs 1-51, wherein said at least one nucleic acid label is modified.
53. The method of any of paragraphs 1-52, wherein said at least one nucleic acid label is designed for minimal cross-hybridization of bases with each other.
54. The method of any of paragraphs 1-53, wherein said at least one nucleic acid label is conjugated to at least one detectable molecule.
55. The method of paragraph 54, wherein said at least one detectable molecule is an optical molecule selected from the group consisting of a small-molecule dye, a fluorescent protein, a quantum dot, a Raman label, a chromophore, and any combinations thereof.
56. The method of any of paragraphs 1-55, wherein each of said plurality of the pre-determined subsequences comprises at least one base.
57. The method of any of paragraphs 1-56, wherein each of said plurality of the pre-determined subsequences comprises from 1 to 100 nucleobases.
58. The method of any of paragraphs 1-57, wherein said plurality of the pre-determined subsequences are conjugated together by at least one sequence linker.
59. The method of paragraph 58, wherein said sequence linker is a bond.
60. The method of any of paragraphs 58-59, wherein said sequence linker is a nucleotidic linker.
61. The method of paragraph 60, wherein said nucleotidic linker is single-stranded, double-stranded, partially double-stranded, a hairpin or any combinations thereof.
62. The method of paragraph 60 or 61, wherein said nucleotidic linker is at least one nucleotide long.
63. The method of any of paragraphs 1-62, wherein said detection reagent comprises one probe reagent and a plurality of nucleic acid labels.

64. The method of any of paragraphs 1-62, wherein said detection reagent comprises a plurality of probe reagents and a nucleic acid label.
65. The method of any of paragraphs 1-62, wherein said detection reagent comprises a plurality of probe reagents and a plurality of nucleic acid labels.
66. The method of any of paragraphs 1-65, wherein the method is adapted for use in immunofluorescence.
67. The method of any of paragraphs 1-66, wherein the method is adapted for use in immunohistochemistry.
68. The method of any of paragraphs 1-67, wherein the method is adapted for use in fluorescence in situ hybridization.
69. The method of any of paragraphs 1-68, wherein the method is adapted for use in western blot.
70. A detection reagent comprising at least one probe reagent and at least one nucleic acid label,
wherein said at least one nucleic acid label comprises at least one pre-determined subsequence to be detected in a temporally-sequential manner;
wherein said at least one pre-determined subsequence forms an identifier of said at least one probe reagent; and
wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together.
71. The detection reagent of paragraph 70, wherein the detection reagent is present in a soluble phase.
72. The detection reagent of paragraph 70 or 71, wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together by at least one linker.
73. The detection reagent of paragraph 72, wherein said linker is a bond.
74. The detection reagent of paragraph 72 or 73, wherein said linker is a linker molecule.

75. The detection reagent of paragraph 74, wherein said linker molecule is a polymer, sugar, nucleic acid, peptide, protein, hydrocarbon, lipid, polyethelyne glycol, crosslinker or combination thereof.
76. The detection reagent of any of paragraphs 72-75, wherein said linker is a particle.
77. The detection reagent of paragraph 76, wherein said particle is selected from a group consisting of a gold nanoparticle, a magnetic bead or nanoparticle, a polystyrene bead, a nanotube, a nanowire, a microparticle, and any combinations thereof.
78. The detection reagent of paragraph 76 or 77, wherein said particle is a nanoparticle.
79. The detection reagent of any of paragraphs 76-78, wherein said particle is modified.
80. The detection reagent of any of paragraphs 76-79, wherein said particle is coated with streptavidin or a derivative thereof.
81. The detection reagent of any of paragraphs 76-80, wherein said particle is modified with at least one functional group.
82. The detection reagent of paragraph 81, wherein the at least one functional group is selected from the group consisting of amine, carboxyl, hydroxyl, aldehyde, ketone, tosyl, silanol, chlorine, hydrazine, hydrazide, photoreactive groups, and any combinations thereof.
83. The detection reagent of any of paragraphs 72-82, wherein said linker is multivalent.
84. The detection reagent of paragraph 83, wherein when the multivalent linker is an avidin-like molecule, both the probe reagent and the nucleic acid label are biotinylated.
85. The detection reagent of any of paragraphs 70-84, wherein said at least one probe reagent is selected from the group consisting of a nucleic acid, an antibody or a portion thereof, an antibody-like molecule, an enzyme, a cell, an antigen, a small molecule, a protein, a peptide, a peptidomimetic, a sugar, a carbohydrate, a lipid, a glycan, a glycoprotein, an aptamer, and any combinations thereof.
86. The detection reagent of any of paragraphs 70-85, wherein said at least one probe reagent is modified.

87. The detection reagent of any of paragraphs 70-86, wherein said at least one probe reagent is biotinylated.
88. The detection reagent of any of paragraphs 70-87, wherein said at least one nucleic acid label is single-stranded, double-stranded, partially double-stranded, a hairpin, linear, circular, branched, a concatemer, or any combinations thereof.
89. The detection reagent of any of paragraphs 70-88, wherein said at least one nucleic acid label is modified.
90. The detection reagent of any of paragraphs 70-89, wherein said at least one nucleic acid label is designed for minimal cross-hybridization of bases with each other.
91. The detection reagent of any of paragraphs 70-90, wherein said at least one nucleic acid label is conjugated to at least one detectable molecule.
92. The detection reagent of paragraph 91, wherein said at least one detectable molecule is an optical molecule selected from the group consisting of small-molecule dye, a fluorescent protein, a quantum dot, a Raman label, a chromophore, and any combinations thereof.
93. The detection reagent of any of paragraphs 70-92, wherein said at least one nucleic acid label comprises a plurality of pre-determined subsequences.
94. The detection reagent of any of paragraphs 70-93, wherein each of said plurality of predetermined subsequences comprises at least one base.
95. The detection reagent of any of paragraphs 70-94, wherein each of said plurality of predetermined subsequences comprises from 1 to 100 nucleobases.
96. The detection reagent of any of paragraphs 70-95, wherein said plurality of the pre-determined subsequences are conjugated together by at least one sequence linker.
97. The detection reagent of paragraph 96, wherein said sequence linker is a bond.
98. The detection reagent of any of paragraphs 96-97, wherein said sequence linker is a nucleotidic linker.
99. The detection reagent of paragraph 98, wherein said nucleotidic linker is single-stranded, double-stranded, partially double-stranded, a hairpin, or any combinations thereof.

100. The detection reagent of paragraph 98 or 99, wherein said nucleotidic linker is at least one nucleotide long.
101. The detection reagent of any of paragraphs 70-100, wherein said detection reagent comprises one probe reagent and a plurality of nucleic acid labels.
102. The detection reagent of any of paragraphs 70-101, wherein said detection reagent comprises a plurality of probe reagents and a nucleic acid label.
103. The detection reagent of any of paragraphs 70-102, wherein said detection reagent comprises a plurality of probe reagents and a plurality of nucleic acid labels.
104. The detection reagent of any of paragraphs 70-103, wherein the detection reagent is adapted for use in immunofluorescence.
105. The detection reagent of any of paragraphs 70-104, wherein the detection reagent is adapted for use in immunohistochemistry.
106. The detection reagent of any of paragraphs 70-105, wherein the detection reagent is adapted for use in fluorescence in situ hybridization.
107. The detection reagent of any of paragraphs 70-106, wherein the detection reagent is adapted for use in western blot.
108. A kit comprising:
 - a. a plurality of the detection reagents of any of paragraphs 70-107; and
 - b. at least one reagent.
109. The kit of paragraph 108, wherein the kit further comprises at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.
110. The kit of paragraph 108 or 109, wherein said detection reagents are present in a soluble phase.
111. The kit of any of paragraphs 108-110, wherein said detection reagents are immobilized in a multi-well plate.

112. The kit of any of paragraphs 108-111, wherein said at least one reagent is selected from the group consisting of a readout reagent, a wash buffer, a signal removal buffer, and any combinations thereof.
113. A kit comprising:
- a. a plurality of the nucleic acid labels of the detection reagents of any of paragraphs 70-107;
 - b. at least one coupling agent that allows a user to conjugate the nucleic acid labels to the user's probe reagents of interest, thereby forming the detection reagents comprising the user's probe reagents of interest; and
 - c. at least one reagent.
114. The kit of paragraph 113, wherein the kit further comprises at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.
115. The kit of paragraph 113 or 114, wherein the detection reagents comprising the user's probe reagents of interest are present in a soluble phase.
116. The kit of any of paragraphs 113-115, wherein the detection reagents comprising the user's probe reagents of interest are immobilized in a multi-well plate.
117. The kit of any of paragraphs 113-116, wherein said at least one reagent is selected from the group consisting of a readout reagent, a wash buffer, a signal removal buffer, and any combinations thereof.

Some selected definitions

[00291] Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. Unless explicitly stated otherwise, or apparent from context, the terms and phrases below do not exclude the meaning that the term or phrase has acquired in the art to which it pertains. The definitions are provided to aid in describing particular embodiments of the aspects described herein, and are not intended to limit the claimed invention, because the scope of the invention is limited only by the claims. Further, unless

otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[00292] As used herein the term “comprising” or “comprises” is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the invention, yet open to the inclusion of unspecified elements, whether essential or not. Additionally, the term “comprising” or “comprises” includes “consisting essentially of” and “consisting of.”

[00293] As used herein the term “consisting essentially of” refers to those elements required for a given embodiment. The term permits the presence of additional elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention.

[00294] The term “consisting of” refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[00295] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term “about.” The term “about” when used in connection with percentages can mean $\pm 1\%$.

[00296] The singular terms “a,” “an,” and “the” include plural referents unless context clearly indicates otherwise. Similarly, the word “or” is intended to include “and” unless the context clearly indicates otherwise.

[00297] Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The term “comprises” means “includes.” The abbreviation, “e.g.” is derived from the Latin *exempli gratia*, and is used herein to indicate a non-limiting example. Thus, the abbreviation “e.g.” is synonymous with the term “for example.”

[00298] As used herein, the term “identifier” generally refers to a unique expression to distinguish variations from one to another among a class of substances, items, or objects. In particular embodiments, the term “identifier” as used herein refers to association of a unique pre-determined subsequence to a specific probe reagent, thus conferring the presence and identity of the probe reagent when the pre-determined subsequence is detected.

[00299] The term “statistically significant” or “significantly” refers to statistical significance and generally means a two standard deviation (2SD) above or below a reference level. The term refers to statistical evidence that there is a difference. It is defined as the probability of making a decision to reject the null hypothesis when the null hypothesis is actually true. The decision is often made using the p-value.

[00300] As used herein, the term “substantially” means a proportion of at least about 60%, or preferably at least about 70% or at least about 80%, or at least about 90%, at least about 95%, at least about 97% or at least about 99% or more, or any integer between 70% and 100%. In some embodiments, the term “substantially” means a proportion of at least about 90%, at least about 95%, at least about 98%, at least about 99% or more, or any integer between 90% and 100%. In some embodiments, the term “substantially” can include 100%.

[00301] The term “sphere” means a particle having an aspect ratio of at most 3:1. The term “aspect ratio” means the ratio of the longest axis of an object to the shortest axis of the object, where the axes are not necessarily perpendicular.

[00302] The term “rod” means a particle having a longest dimension of at most 5000 nm, and having an aspect ratio of from 3:1 to 20:1.

[00303] The term “prism” means a particle having at least two non-parallel faces connected by a common edge.

[00304] As used herein, the term “pharmaceutically acceptable carrier” refers to a pharmaceutically-acceptable material, composition or vehicle for administration of the detection reagents. Pharmaceutically acceptable carriers include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like which are compatible with the activity of the detection reagents and are physiologically acceptable to the subject. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, methylcellulose, ethyl cellulose, microcrystalline cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) lubricating agents, such as magnesium stearate, sodium lauryl sulfate and talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and

polyethylene glycol (PEG); (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) pH buffered solutions; (21) polyesters, polycarbonates and/or polyanhydrides; (22) bulking agents, such as polypeptides and amino acids (23) serum component, such as serum albumin, HDL and LDL; (22) C2-C12 alcohols, such as ethanol; and (23) other non-toxic compatible substances employed in pharmaceutical formulations. Wetting agents, coloring agents, release agents, coating agents, sweetening agents, flavoring agents, perfuming agents, preservative and antioxidants can also be present in the formulation. The terms such as "excipient", "carrier", "pharmaceutically acceptable carrier" are used interchangeably herein.

[00305] As used here, the term "pharmaceutically acceptable" refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[00306] As used herein, the term "therapeutic agent" refers to a biological or chemical agent used for treatment, curing, mitigating, or preventing deleterious conditions in a subject. The term "therapeutic agent" also includes substances and agents for combating a disease, condition, or disorder of a subject, and includes drugs, diagnostics, and instrumentation. "Therapeutic agent" also includes anything used in medical diagnosis, or in restoring, correcting, or modifying physiological functions. The terms "therapeutic agent" and "pharmaceutically active agent" are used interchangeably herein.

[00307] A therapeutic agent can be selected according to the treatment objective and biological action desired. Thus, a therapeutic agent can be selected from any class suitable for the therapeutic objective. Further, the therapeutic agent may be selected or arranged to provide therapeutic activity over a period of time.

[00308] Exemplary pharmaceutically active compound include, but are not limited to, those found in Harrison's Principles of Internal Medicine, 13th Edition, Eds. T.R. Harrison McGraw-Hill N.Y., NY; Physicians Desk Reference, 50th Edition, 1997, Oradell New Jersey, Medical Economics Co.; Pharmacological Basis of Therapeutics, 8th Edition, Goodman and Gilman, 1990; United States Pharmacopeia, The National Formulary, USP XII NF XVII, 1990; current

edition of Goodman and Oilman's The Pharmacological Basis of Therapeutics; and current edition of The Merck Index, the complete content of all of which are herein incorporated in its entirety.

[00309] Exemplary pharmaceutically active agents include, but are not limited to, steroids and nonsteroidal anti-inflammatory agents, antirestenotic drugs, antimicrobial agents, angiogenic factors, calcium channel blockers, thrombolytic agents, antihypertensive agents, anti-coagulants, antiarrhythmic agents, cardiac glycosides, and the like.

[00310] In some embodiments, the therapeutic agent is selected from the group consisting of salicylic acid and derivatives (aspirin), para-aminophenol and derivatives (acetaminophen), arylpropionic acids (ibuprofen), corticosteroids, histamine receptor antagonists and bradykinin receptor antagonists, leukotriene receptor antagonists, prostaglandin receptor antagonists, platelet activating factor receptor antagonists, sulfonamides, trimethoprim-sulfamethoxazole, quinolones, penicillins, cephalosporin, basic fibroblast growth factor (FGF), acidic fibroblast growth factor, vascular endothelial growth factor, angiogenic transforming growth factor alpha and beta, tumor necrosis factor, angiopoietin, platelet-derived growth factor, dihydropyridines (e.g., nifedipine, benzothiazepines such as diltiazem, and phenylalkylamines such as verapamil), urokinase plasminogen activator, urokinase, streptokinase, angiotensin converting enzyme (ACE) inhibitors, spironolactone, tissue plasminogen activator (tPA), diuretics, thiazides, antiadrenergic agents, clonidine, propranolol, angiotensin-converting enzyme inhibitors, captopril, angiotensin receptor antagonists, losartan, calcium channel antagonists, nifedine, heparin, warfarin, hirudin, tick anti-coagulant peptide, and low molecular weight heparins such as enoxaparin, lidocaine, procainamide, encainide, flecanide, beta adrenergic blockers, propranolol, amiodarone, verpamil, diltiazem, nickel chloride, cardiac glycosides, angiotensin converting enzyme inhibitors, angiotensin receptor antagonists, nitrovasodilators, hypolipidemic agents (e.g., nicotinic acid, probucol, etc.), bile acid-binding resins (e.g., cholestyramine, and fibric acid derivatives e.g., clofibrate), HMG CoA reductase inhibitors, HMG CoA synthase inhibitors, squalene synthase inhibitors, squalene epoxidase inhibitors, statins (e.g., lovastatin, cerivastatin, fluvastatin, pravastatin, simvastatin, etc.), anti-psychotics, SSRIs, antiseizure medication, contraceptives, systemic and local analgesics (chronic pain, bone growth/remodeling factors (osteoblast/osteoclast recruiting and stimulating factors), neurotransmitters (L-DOPA, Dopamine, neuropeptides), emphysema drugs, TGF-beta), rapamycin, naloxone, paclitaxel,

amphotericin, Dexamethasone, flutamide, vancomycin, phenobarbital, cimetidine, atenolol, aminoglycosides, hormones (e.g., thyrotropin-releasing hormone, p-nitrophenyl beta-cellopentaoside and luteinizing hormone-releasing hormone), vincristine, amiloride, digoxin, morphine, procainamide, quinidine, quinine, ranitidine, triamterene, trimethoprim, vancomycin, aminoglycosides, and penicillin, and pharmaceutically acceptable salts thereof.

[00311] As used herein, the term “fluorescent color” refers to a color emitted by any fluorophore (e.g., fluorescent dye, fluorescent particles such as quantum dots, and/or fluorescent proteins) upon light excitation and/or electromagnetic radiation. Fluorescent dye as disclosed herein refers to a dye which exhibits energy and is visible under illumination at a predefined wavelength. Numerous fluorescent molecules are commercially available and can be adapted for use in the methods, detection reagents and kits as disclosed herein, and include those from Molecular Probes, Sigma and similar other commercial sources. In some embodiments, a “fluorescent color” can be produced by phosphorescence. In some embodiments, a “fluorescent color” can be produced by luminescence (including bioluminescence).

[00312] To the extent not already indicated, it will be understood by those of ordinary skill in the art that any one of the various embodiments herein described and illustrated may be further modified to incorporate features shown in any of the other embodiments disclosed herein.

[00313] The following examples illustrate some embodiments and aspects of the invention. It will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be performed without altering the spirit or scope of the invention, and such modifications and variations are encompassed within the scope of the invention as defined in the claims which follow. The following examples do not in any way limit the invention.

EXAMPLES

Example 1: Exemplary hybridization-based detection methods of detection reagents

[00314] A solution suspension of streptavidin-coated Dynabeads (of about 1 μm), each conjugated to one of six nucleic acid labels and a corresponding probe reagent specific for a target analyte, wherein each of the nucleic acid labels comprised at least one pre-determined subsequence, e.g., a first (e.g., A1, A2, and A3) and a second (B1, B2, and B3) pre-determined subsequence, was prepared. The nucleic acid labels can comprise a nucleic acid sequence of any

length. In some embodiments, the nucleic acid labels can comprise at least 15 nucleotides, at least 20 nucleotides, at least 22 nucleotides, or at least 24 nucleotides. A sample containing multiple target analytes was contacted with the target analyte-specific Dynabeads, so that the Dynabeads would bind to the respective target analyte on the sample. Any unbound Dynabeads was then removed, e.g., by washing. In the first readout, a set of three decoder probes, each with a distinct sequence complementary to the first pre-determined subsequences (e.g., A1*, A2*, and A3*) and a corresponding optical label (e.g., selected from red, green or blank), was added to the sample with bound Dynabeads, and then imaged with fluorescent microscopy (**Fig. 6A**). The fluorescent signal was then removed, e.g., by photobleaching, from the previous stage before the next readout. In the second readout, a different set of three decoder probes, each with a distinct complementary sequence to the second pre-determined subsequences (e.g., B1*, B2*, B3*) and a corresponding optical label (e.g., selected from red, green blank), was added to the photobleached sample, and then imaged with fluorescent microscopy (**Fig. 6B**). The temporal sequence of the optical signatures obtained from each bead can then be analyzed to determine the identity of the probe and thus the presence of the corresponding target analyte on the sample. Based on the intensity and/or coverage of the optical signals, the amount of the target analyte can also be determined.

[00315] By way of example only, a biotinylated anti-*C. albicans* antibody (e.g., an commercially-available antibody from Pierce Antibodies PA1-27145) was conjugated with a plurality of different biotinylated nucleic acid labels (e.g., 8 different biotinylated SeqTag label sequences) using any conjugation methods known in the art. In one embodiment, the biotinylated anti-*C. albicans* antibody was conjugated with a plurality of different biotinylated nucleic acid labels (e.g., biotinylated SeqTag label sequences) using a streptavidin-like protein (e.g., Neutravidin) as a bridge. The SeqTag label sequences are shown in Table 5. The SeqTag label sequences can comprise at least about 24 nucleotides. In some embodiments, the SeqTag label sequences can comprise DNA, RNA or a combination thereof. Each conjugate was incubated with a sample comprising *C. albicans*, washed, and readout using any readout method as described herein, e.g., the displacement-hybridization method.

Table 5. DNA sequences of exemplary SeqTag labels

SEQ ID NO:	SeqTag label	DNA Sequence
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59	SeqTag 1	5'-Biotin – TTTCGCTTTCTGTAATGGAGTGGA – 3'
60	SeqTag 2	5'-Biotin – TTTCGCTTTAGCCTAAGTGAAATC – 3'
61	SeqTag 3	5'-Biotin – TTTCGCTTTTTTGGGGAAAAGACA – 3'
62	SeqTag 4	5'-Biotin – TTTCGCTTTTAGGCATTAGCATTG – 3'
63	SeqTag 5	5'-Biotin – TTTCGCTTTGGAAGCACCTATTCC – 3'
64	SeqTag 6	5'-Biotin – TTTCGCTTTCTGGAGAAAGGGCCA – 3'
65	SeqTag 7	5'-Biotin – TTTCGCTTTCGGTTCCAAAGACAC – 3'
66	SeqTag 8	5'-Biotin – TTTCGCTTTGAAGCCGGTTATAGC – 3'

[00316] During the displacement hybridization method, by way of example only, as shown in **Fig. 7**, the yeast was incubated in readout step 1 with a mixture of all three “set 1” decoder probes, followed by an incubation with a mixture of the “set 2” decoder probes and the “set 1 displacers” (which are nucleic acid sequences to remove “set 1” fluorescence), and similarly with appropriate sets of decoder probes and displacers for steps 3 and 4. The decoder probes can comprise a nucleic acid sequence (e.g., DNA, RNA or a combination thereof) and a detection label, e.g., at its 5' end. The nucleic acid sequence of the decoder probes can be of any length. In some embodiments, the decoder probes can comprise at least about 10 nucleotides, at least about 12 nucleotides, at least about 14 nucleotides, at least about 16 nucleotides, at least about 18 nucleotides, at least about 20 nucleotides or longer. Detection labels can be any art-recognized label described herein, e.g., fluorescent detection labels such as FAM, Cy3, and C5, or any labels described herein. Probe displacers, as described earlier, are nucleic acid sequences that are used to remove or displace the previous decoder probes hybridized to the nucleic acid labels (e.g., SeqTag labels) such that a different set of decoder probes can be added and hybridized to the nucleic acid labels. The probe displacers can have a nucleic acid sequence of any length. In some embodiments, the nucleic acid sequence of the probe displacers can be longer than that of the decoder probes. In some embodiments, the probe displacers can comprise at least about 12 nucleotides, at least about 14 nucleotides, at least about 16 nucleotides, at least about 18 nucleotides, at least about 20 nucleotides, at least about 21 nucleotides or longer. Tables 2 and 3 show DNA sequences of exemplary decoder probes (or readout probes) and probe displacers, respectively. As seen in **Fig. 7**, each biotinylated anti-*C. albicans* antibody conjugated with a different SeqTag label sequence properly stained the yeast and fluoresced only as designated by its SeqTag label with appropriate sets of decoder probes and probe displacer in each readout step.

Table 6. DNA sequences of exemplary decoder probes

SEQ ID NO:	Readout probe	DNA Sequence
67	Set 1 FAM	5'-FAM – TTTTCCACTCCATTACAG – 3'
68	Set 1 Cy3	5'-Cy3 – TTTGATTTCACCTAGGCT – 3'
69	Set 1 Cy5	5'-Cy5 – TTTTGTCTTTTCCCCAAA – 3'
70	Set 2 FAM	5'-FAM – TTTCAATGCTAATGCCTA – 3'
71	Set 2 Cy3	5'-Cy3 – TTTGGAATAGGTGCTTCC – 3'
72	Set 2 Cy5	5'-Cy5 – TTTTGGCCCTTTCTCCAG – 3'
73	Set 3 FAM	5'-FAM – TTTGTGTCTTTGGAACCG – 3'
74	Set 3 Cy3	5'-Cy3 – TTTGCTATAACCGGCTTC – 3'

Table 7. DNA sequences of exemplary probe displacers

SEQ ID NO:	Probe Displacer	DNA Sequence
75	Set 1 FAM-Displacer	5' – TCCACTCCATTACAGAAAGCG – 3'
76	Set 1 Cy3-Displacer	5' – GATTTCACCTAGGCTAAAGCG – 3'
77	Set 1 Cy5-Displacer	5' – TGTCTTTTCCCCAAAAAAGCG – 3'
78	Set 2 FAM-Displacer	5' – CAATGCTAATGCCTAAAAGCG – 3'
79	Set 2 Cy3-Displacer	5' – GGAATAGGTGCTTCCAAAGCG – 3'
80	Set 2 Cy5-Displacer	5' – TGGCCCTTTCTCCAGAAAGCG – 3'
81	Set 3 FAM-Displacer	5' – TGTGTCTTTGGAACCGAAAGCG – 3'
82	Set 3 Cy3-Displacer	5' – GCTATAACCGGCTTCAAAGCG – 3'

[00317] Content of all patents and other publications identified herein is expressly incorporated herein by reference for all purposes. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

CLAIMS

What is claimed is:

1. A method for detecting a plurality of analytes in a sample, comprising:
 - a. contacting the sample with a composition comprising a plurality of detection reagents, wherein each subpopulation of the detection reagents targets at least one different analyte, and wherein each detection reagent comprises:

at least one probe reagent targeting an analyte and at least one nucleic acid label comprising one or a plurality of pre-determined subsequences, wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together; and wherein at least a portion of said one or the plurality of pre-determined subsequences form an identifier of said at least one probe reagent; and
 - b. detecting in a temporally-sequential manner said one or the plurality of the pre-determined subsequences of said detection reagent, wherein said detection of the subsequences each generates a signal signature corresponding to said subsequence, and wherein a temporal order of the signal signatures corresponding to said one or the plurality of the subsequences of said detection reagent identifies a subpopulation of the detection reagents.
2. The method of claim 1, wherein said each subpopulation of the detection reagents targets a set of analytes.
3. The method of claim 1 or 2, wherein the temporal order of the signal signatures corresponding to said one or the plurality of the subsequences of said detection reagent is unique for each subpopulation of the detection reagents.
4. The method of any of claims 1-3, wherein said detection reagents are present in a soluble phase.
5. The method of any of claims 1-4, further comprising processing said sample before said contacting with said plurality of detection reagents.
6. The method of any of claims 1-5, further comprising removing any unbound detection reagents before the detecting step (b).

7. The method of any of claims 1-6, further comprising comparing said temporal order of the signal signatures with different identifiers of said at least one probe reagent, wherein an agreement between the temporal order of the signal signatures and a particular identifier of said at least one probe reagent identifies the analyte in the sample.
8. The method of any of claims 1-7, further comprising measuring the intensity of the signal signatures generated from each subpopulation of the detection reagents.
9. The method of claim 8, wherein the intensity of the signal signatures generated from each subpopulation of the detection reagents indicates an amount of the analyte.
10. The method of claim 8 or 9, wherein the intensity of the signal signatures generated from each subpopulation of the detection reagents is used in identification of the subpopulation of the detection reagents.
11. The method of any of claims 1-10, wherein said detecting of step (b) comprises sequencing.
12. The method of claim 11, wherein said sequencing is performed via ligation, hybridization, synthesis, amplification, or single-base extension.
13. The method of any of claims 1-12 wherein said detecting of step (b) comprises hybridizing a decoder probe with said subsequence, wherein said decoder probe comprises a detectable label.
14. The method of any of claims 1-13, wherein said detecting of step (b) comprises:
 - a. hybridizing a set of decoder probes with a subsequence of the detection reagents, wherein each subpopulation of the decoder probes comprises a detectable label, each detectable label producing a signal signature;
 - b. detecting said signal signature produced by the hybridization of said set of decoder probes;
 - c. optionally removing said different signal signature produced by the hybridization of said set of decoder probes; and

- d. repeating steps (a) through (c) for other subsequences of said detection reagents, thereby producing a temporal order of the signal signatures corresponding to said each detection reagent.
15. The method of claim 14, wherein said each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.
 16. The method of claim 14 or 15, wherein said each subpopulation of the decoder probes is at least partially or completely complementary to said subsequence of the detection reagents.
 17. The method of any of claims 14-16, wherein at least two or more subpopulations of the decoder probes are at least partially or completely complementary to the same subsequence of the detection reagents.
 18. The method of any of claims 14-17, wherein said removing step is performed by washing, heating, photobleaching, displacement, cleavage, enzymatic digestion, quenching, chemical degradation, bleaching, oxidation or any combinations thereof.
 19. The method of any of claims 14-18, wherein said detectable label comprises or is an optical label selected from the group consisting of a small-molecule dye, a fluorescent molecule, a fluorescent protein, a quantum dot, Raman label, a chromophore, and any combinations thereof.
 20. The method of any of claims 14-19, wherein said detectable label comprises or is a colorimetric reagent.
 21. The method of any of claims 14-20, wherein said detectable label comprises or is a Raman label.
 22. The method of any of claims 1-21, wherein said signal signatures comprise or are optical signatures.
 23. The method of claim 22, wherein said optical signatures comprise signatures of fluorescent color, visible light, no-color, Raman label, or any combinations thereof.

24. The method of claim 22, wherein said optical signatures comprise signatures of one or more fluorescent colors, one or more visible lights, one or more no-colors, one or more Raman labels, or any combinations thereof.
25. The method of any of claims 22-24, wherein said optical signatures are detected by optical imaging or spectroscopy.
26. The method of any of claims 1-25, wherein said analytes are selected from the group consisting of antigens, receptors, proteins, peptides, sugars, glycoproteins, peptidoglycans, lipids, nucleic acids, oligonucleotides, cells, viruses, and any combinations thereof.
27. The method of any of claims 1-26, wherein said nucleic acids are selected from the group consisting of cellular DNA or RNA, messenger RNA, microRNA, ribosomal RNA, and any combinations thereof.
28. The method of any of claims 1-27, wherein said sample is a protein sample immobilized on a solid support.
29. The method of claim 28, wherein the solid support is a blotting membrane.
30. The method of any of claims 1-27, wherein said sample is a biological sample.
31. The method of claim 30, wherein said biological sample comprises one or more cells, one or more tissues, one or more fluids or any combinations thereof.
32. The method of claim 30 or 31, wherein said biological sample comprises blood, sputum, cerebrospinal fluid, urine, saliva, sperm, sweat, mucus, nasal discharge, vaginal fluids or any combinations thereof.
33. The method of any of claims 30-31, wherein the said biological sample comprises a biopsy, a surgically removed tissue, a swap or any combinations thereof.
34. The method of claims 1-27, wherein said sample comprises an environmental sample, food, food byproduct, soil, an archaeological sample, an extraterrestrial sample, or any combinations thereof.
35. The method of any of claims 1-34, wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together by at least one linker.

36. The method of claim 35, wherein said linker is a bond.
37. The method of claim 35 or 36, wherein said linker is a linker molecule.
38. The method of claim 37, wherein said linker molecule is a polymer, sugar, nucleic acid, peptide, protein, hydrocarbon, lipid, polyethylene glycol, crosslinker, or any combinations thereof.
39. The method of any of claims 35-38, wherein said linker is a particle.
40. The method of claim 39, wherein said particle is selected from a group consisting of a gold nanoparticle, a magnetic bead or nanoparticle, a polystyrene bead, a nanotube, a nanowire, a microparticle, and any combinations thereof.
41. The method of claim 40, wherein said particle is a nanoparticle.
42. The method of any of claims 39-41, wherein said particle is modified.
43. The method of any of claims 39-42, wherein said particle is coated with streptavidin or a derivative thereof.
44. The method of any of claims 39-43, wherein said particle is modified with at least one functional group.
45. The method of claim 44, wherein said at least one functional group is selected from the group consisting of amine, carboxyl, hydroxyl, aldehyde, ketone, tosyl, silanol, chlorine, hydrazine, hydrazide, photoreactive groups, and any combinations thereof.
46. The method of any of claims 35-45, wherein said linker is multivalent.
47. The method of claim 46, wherein when the multivalent linker is an avidin-like molecule, both the probe reagent and the nucleic acid label are biotinylated.
48. The method of any of claims 1-47, wherein said at least one probe reagent is selected from the group consisting of a nucleic acid, an antibody or a portion thereof, an antibody-like molecule, an enzyme, a cell, an antigen, a small molecule, a protein, a peptide, a peptidomimetic, a sugar, a carbohydrate, a lipid, a glycan, a glycoprotein, an aptamer, and any combinations thereof.
49. The method of any of claims 1-48, wherein said at least one probe reagent is modified.

50. The method of any of claims 1-49, wherein said at least one probe reagent is biotinylated.
51. The method of any of claims 1-50, wherein said at least one nucleic acid label is single-stranded, double-stranded, partially double-stranded, a hairpin, linear, circular, branched, a concatemer, or any combinations thereof.
52. The method of any of claims 1-51, wherein said at least one nucleic acid label is modified.
53. The method of any of claims 1-52, wherein said at least one nucleic acid label is designed for minimal cross-hybridization of bases with each other.
54. The method of any of claims 1-53, wherein said at least one nucleic acid label is conjugated to at least one detectable molecule.
55. The method of claim 54, wherein said at least one detectable molecule is an optical molecule selected from the group consisting of a small-molecule dye, a fluorescent protein, a quantum dot, a Raman label, a chromophore, and any combinations thereof.
56. The method of any of claims 1-55, wherein each of said plurality of the pre-determined subsequences comprises at least one base.
57. The method of any of claims 1-56, wherein each of said plurality of the pre-determined subsequences comprises from 1 to 100 nucleobases.
58. The method of any of claims 1-57, wherein said plurality of the pre-determined subsequences are conjugated together by at least one sequence linker.
59. The method of claim 58, wherein said sequence linker is a bond.
60. The method of any of claims 58-59, wherein said sequence linker is a nucleotidic linker.
61. The method of claim 60, wherein said nucleotidic linker is single-stranded, double-stranded, partially double-stranded, a hairpin or any combinations thereof.
62. The method of claim 60 or 61, wherein said nucleotidic linker is at least one nucleotide long.
63. The method of any of claims 1-62, wherein said detection reagent comprises one probe reagent and a plurality of nucleic acid labels.

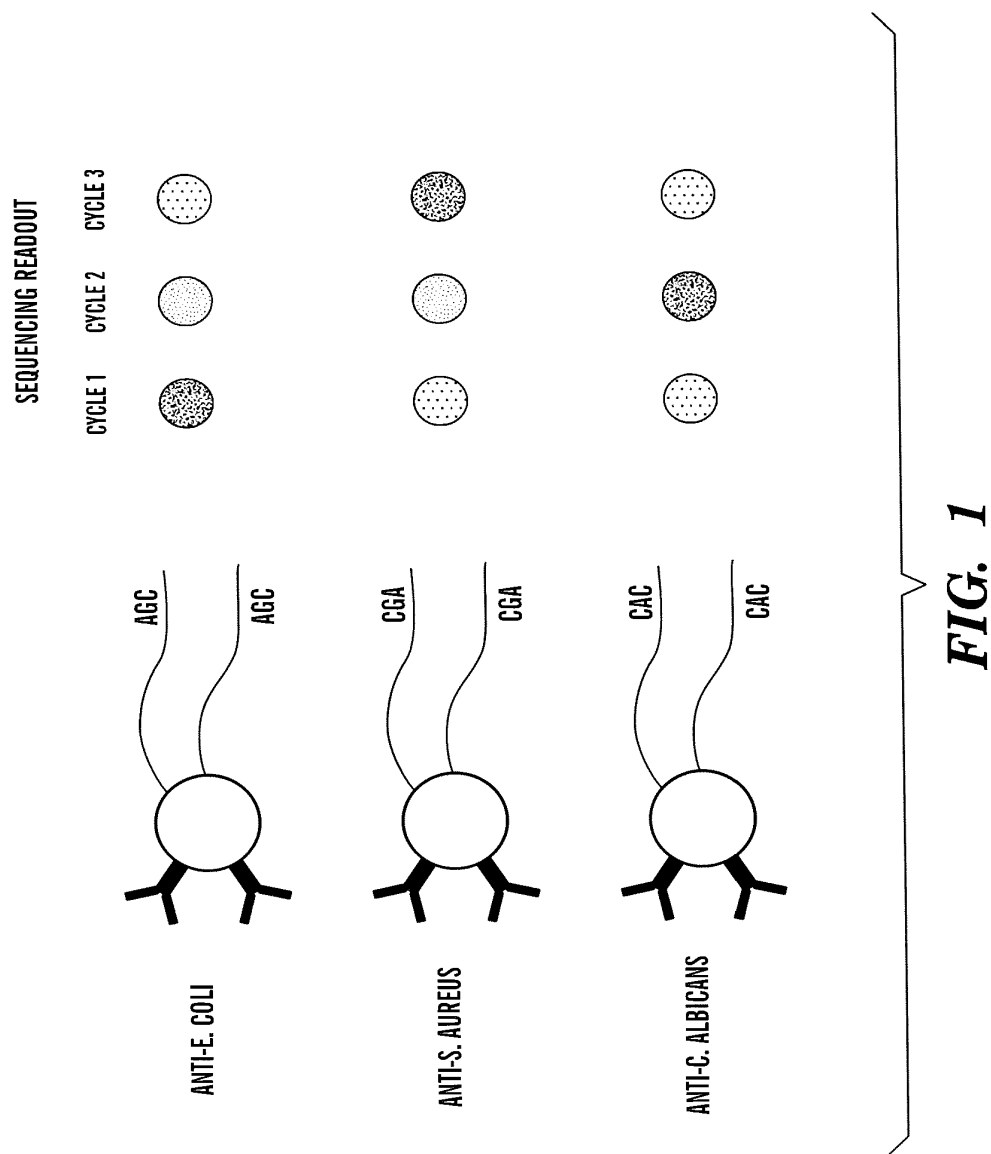
64. The method of any of claims 1-62, wherein said detection reagent comprises a plurality of probe reagents and a nucleic acid label.
65. The method of any of claims 1-62, wherein said detection reagent comprises a plurality of probe reagents and a plurality of nucleic acid labels.
66. The method of any of claims 1-65, wherein the method is adapted for use in immunofluorescence.
67. The method of any of claims 1-66, wherein the method is adapted for use in immunohistochemistry.
68. The method of any of claims 1-67, wherein the method is adapted for use in fluorescence in situ hybridization.
69. The method of any of claims 1-68, wherein the method is adapted for use in western blot.
70. A detection reagent comprising at least one probe reagent and at least one nucleic acid label,
wherein said at least one nucleic acid label comprises at least one pre-determined subsequence to be detected in a temporally-sequential manner;
wherein said at least one pre-determined subsequence forms an identifier of said at least one probe reagent; and
wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together.
71. The detection reagent of claim 70, wherein the detection reagent is present in a soluble phase.
72. The detection reagent of claim 70 or 71, wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together by at least one linker.
73. The detection reagent of claim 72, wherein said linker is a bond.
74. The detection reagent of claim 72 or 73, wherein said linker is a linker molecule.

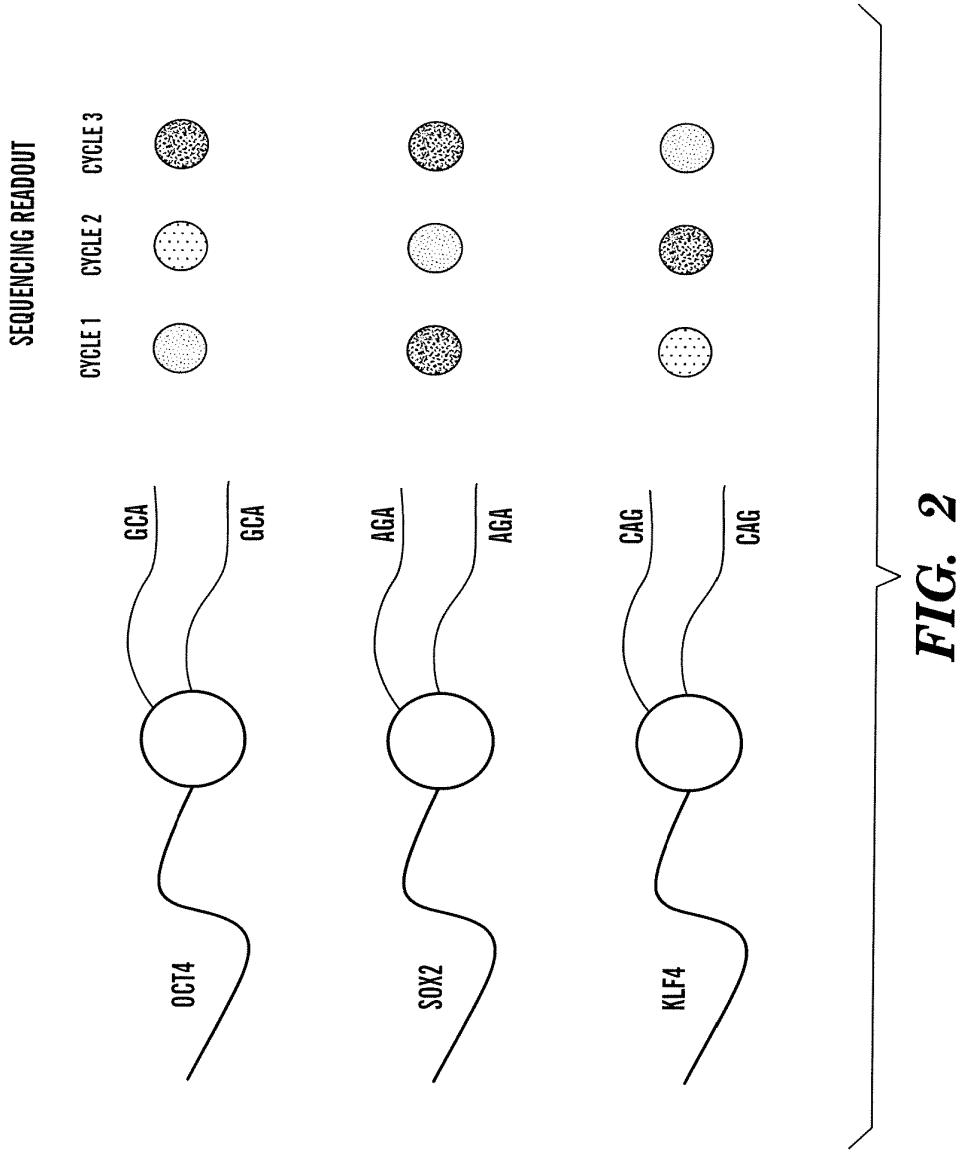
75. The detection reagent of claim 74, wherein said linker molecule is a polymer, sugar, nucleic acid, peptide, protein, hydrocarbon, lipid, polyethylene glycol, crosslinker or combination thereof.
76. The detection reagent of any of claims 72-75, wherein said linker is a particle.
77. The detection reagent of claim 76, wherein said particle is selected from a group consisting of a gold nanoparticle, a magnetic bead or nanoparticle, a polystyrene bead, a nanotube, a nanowire, a microparticle, and any combinations thereof.
78. The detection reagent of claim 76 or 77, wherein said particle is a nanoparticle.
79. The detection reagent of any of claims 76-78, wherein said particle is modified.
80. The detection reagent of any of claims 76-79, wherein said particle is coated with streptavidin or a derivative thereof.
81. The detection reagent of any of claims 76-80, wherein said particle is modified with at least one functional group.
82. The detection reagent of claim 81, wherein the at least one functional group is selected from the group consisting of amine, carboxyl, hydroxyl, aldehyde, ketone, tosyl, silanol, chlorine, hydrazine, hydrazide, photoreactive groups, and any combinations thereof.
83. The detection reagent of any of claims 72-82, wherein said linker is multivalent.
84. The detection reagent of claim 83, wherein when the multivalent linker is an avidin-like molecule, both the probe reagent and the nucleic acid label are biotinylated.
85. The detection reagent of any of claims 70-84, wherein said at least one probe reagent is selected from the group consisting of a nucleic acid, an antibody or a portion thereof, an antibody-like molecule, an enzyme, a cell, an antigen, a small molecule, a protein, a peptide, a peptidomimetic, a sugar, a carbohydrate, a lipid, a glycan, a glycoprotein, an aptamer, and any combinations thereof.
86. The detection reagent of any of claims 70-85, wherein said at least one probe reagent is modified.
87. The detection reagent of any of claims 70-86, wherein said at least one probe reagent is biotinylated.

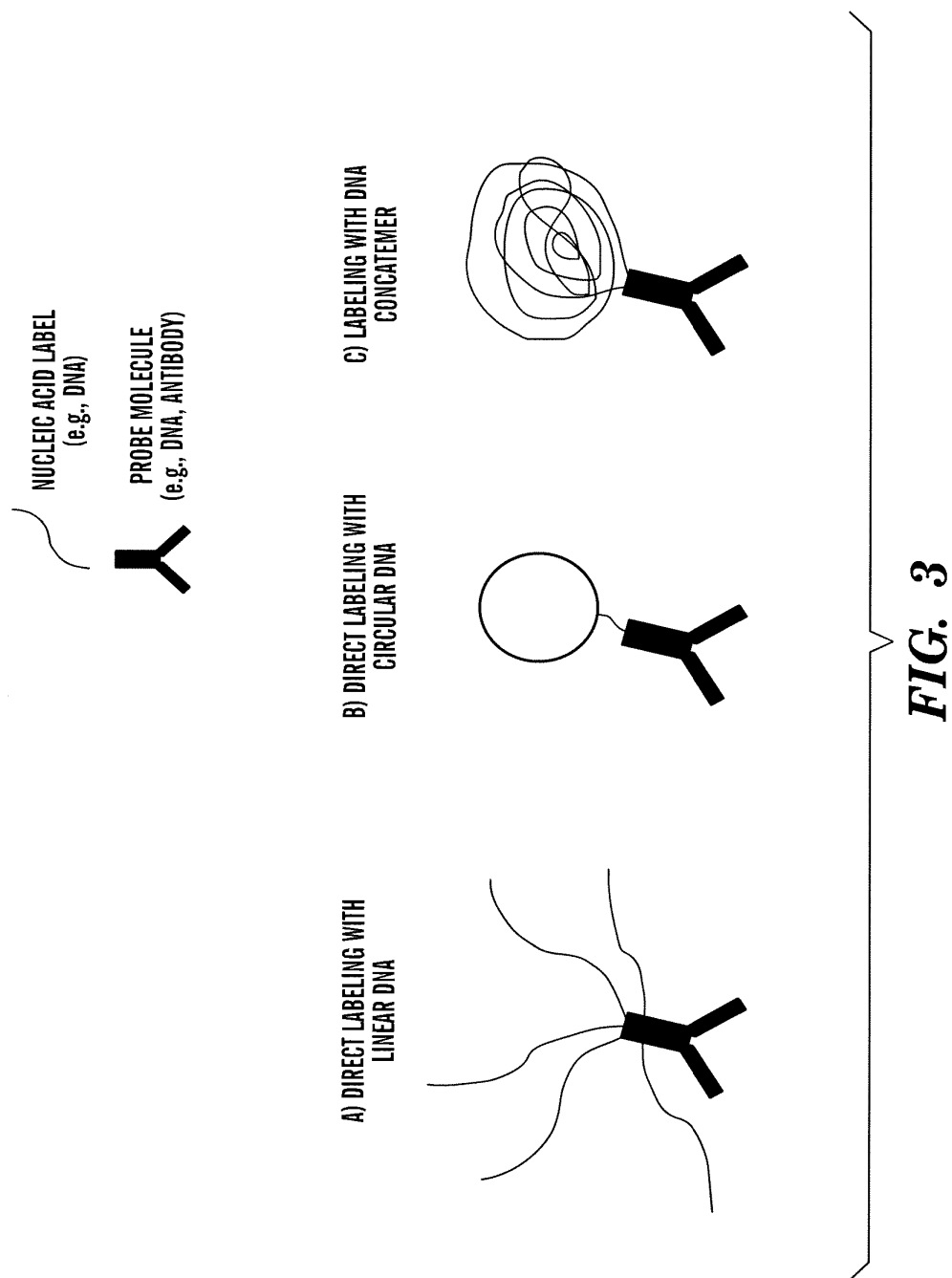
88. The detection reagent of any of claims 70-87, wherein said at least one nucleic acid label is single-stranded, double-stranded, partially double-stranded, a hairpin, linear, circular, branched, a concatemer, or any combinations thereof.
89. The detection reagent of any of claims 70-88, wherein said at least one nucleic acid label is modified.
90. The detection reagent of any of claims 70-89, wherein said at least one nucleic acid label is designed for minimal cross-hybridization of bases with each other.
91. The detection reagent of any of claims 70-90, wherein said at least one nucleic acid label is conjugated to at least one detectable molecule.
92. The detection reagent of claim 91, wherein said at least one detectable molecule is an optical molecule selected from the group consisting of small-molecule dye, a fluorescent protein, a quantum dot, a Raman label, a chromophore, and any combinations thereof.
93. The detection reagent of any of claims 70-92, wherein said at least one nucleic acid label comprises a plurality of pre-determined subsequences.
94. The detection reagent of any of claims 70-93, wherein each of said plurality of predetermined subsequences comprises at least one base.
95. The detection reagent of any of claims 70-94, wherein each of said plurality of predetermined subsequences comprises from 1 to 100 nucleobases.
96. The detection reagent of any of claims 70-95, wherein said plurality of the pre-determined subsequences are conjugated together by at least one sequence linker.
97. The detection reagent of claim 96, wherein said sequence linker is a bond.
98. The detection reagent of any of claims 96-97, wherein said sequence linker is a nucleotidic linker.
99. The detection reagent of claim 98, wherein said nucleotidic linker is single-stranded, double-stranded, partially double-stranded, a hairpin, or any combinations thereof.
100. The detection reagent of claim 98 or 99, wherein said nucleotidic linker is at least one nucleotide long.

101. The detection reagent of any of claims 70-100, wherein said detection reagent comprises one probe reagent and a plurality of nucleic acid labels.
102. The detection reagent of any of claims 70-101, wherein said detection reagent comprises a plurality of probe reagents and a nucleic acid label.
103. The detection reagent of any of claims 70-102, wherein said detection reagent comprises a plurality of probe reagents and a plurality of nucleic acid labels.
104. The detection reagent of any of claims 70-103, wherein the detection reagent is adapted for use in immunofluorescence.
105. The detection reagent of any of claims 70-104, wherein the detection reagent is adapted for use in immunohistochemistry.
106. The detection reagent of any of claims 70-105, wherein the detection reagent is adapted for use in fluorescence in situ hybridization.
107. The detection reagent of any of claims 70-106, wherein the detection reagent is adapted for use in western blot.
108. A kit comprising:
 - a. a plurality of the detection reagents of any of claims 70-107; and
 - b. at least one reagent.
109. The kit of claim 108, wherein the kit further comprises at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.
110. The kit of claim 108 or 109, wherein said detection reagents are present in a soluble phase.
111. The kit of any of claims 108-110, wherein said detection reagents are immobilized in a multi-well plate.
112. The kit of any of claims 108-111, wherein said at least one reagent is selected from the group consisting of a readout reagent, a wash buffer, a signal removal buffer, and any combinations thereof.

113. A kit comprising:
- a. a plurality of the nucleic acid labels of the detection reagents of any of claims 70-107;
 - b. at least one coupling agent that allows a user to conjugate the nucleic acid labels to the user's probe reagents of interest, thereby forming the detection reagents comprising the user's probe reagents of interest; and
 - c. at least one reagent.
114. The kit of claim 113, wherein the kit further comprises at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.
115. The kit of claim 113 or 114, wherein the detection reagents comprising the user's probe reagents of interest are present in a soluble phase.
116. The kit of any of claims 113-115, wherein the detection reagents comprising the user's probe reagents of interest are immobilized in a multi-well plate.
117. The kit of any of claims 113-116, wherein said at least one reagent is selected from the group consisting of a readout reagent, a wash buffer, a signal removal buffer, and any combinations thereof.









BOTH LABELS AND PROBES
CONJUGATED TO PARTICLE

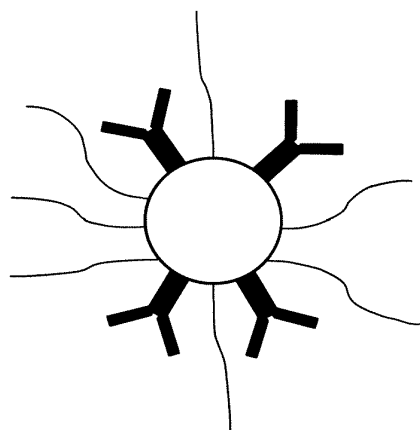


FIG. 4A

PROBES CONJUGATED TO
PARTICLE THROUGH LABELS

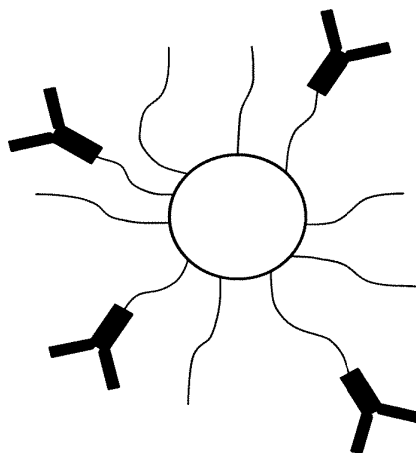
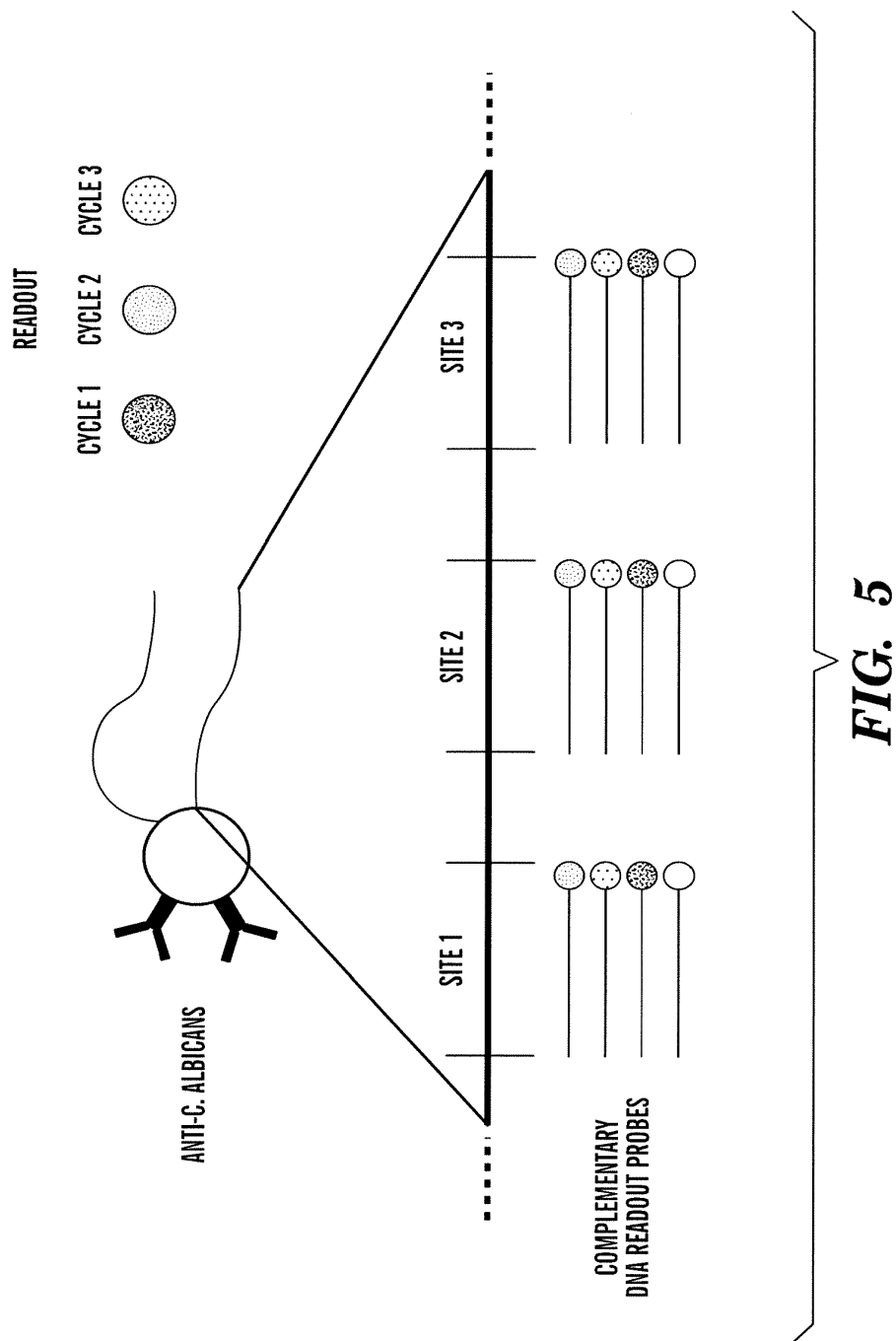
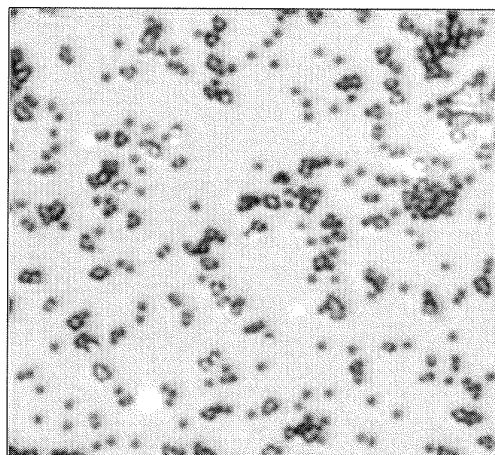


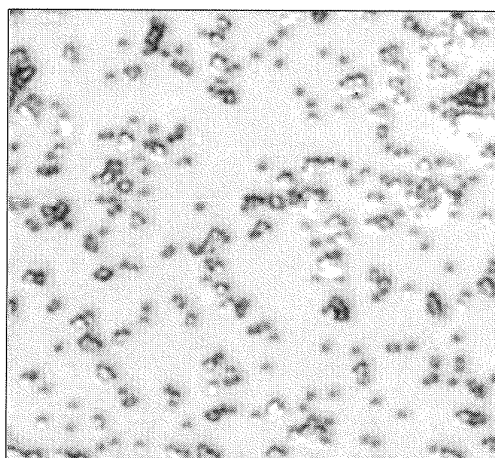
FIG. 4B





READOUT STAGE 2

FIG. 6B



READOUT STAGE 1

FIG. 6A

SUBSTITUTE SHEET (RULE 26)

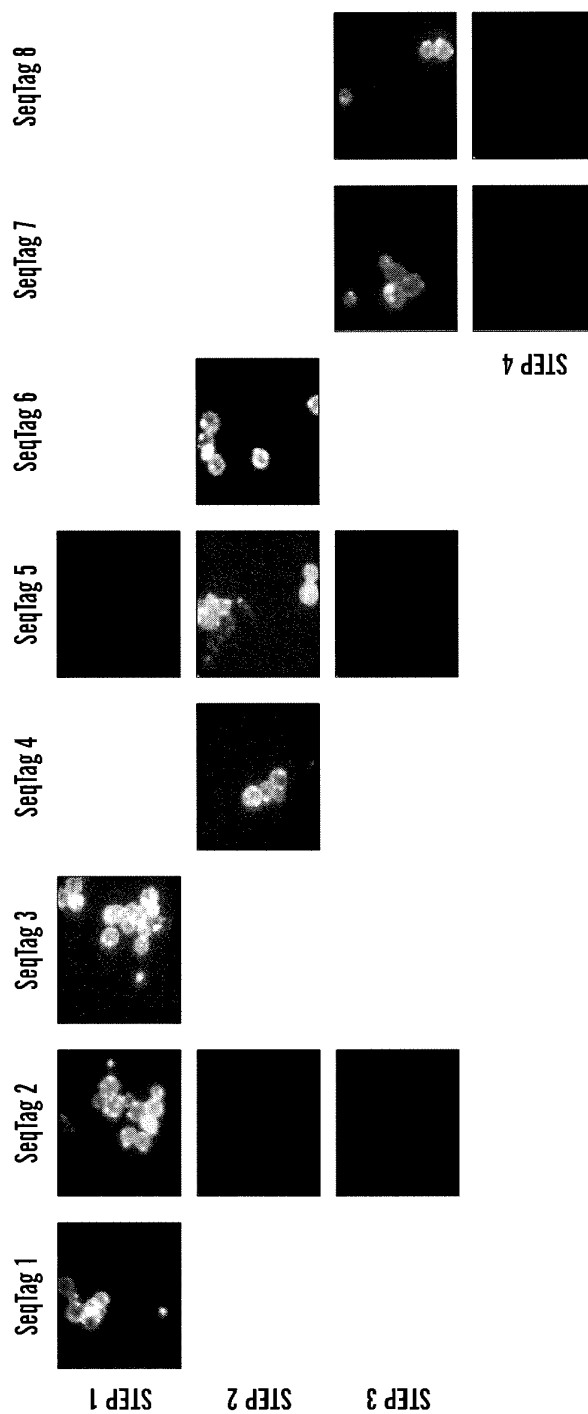
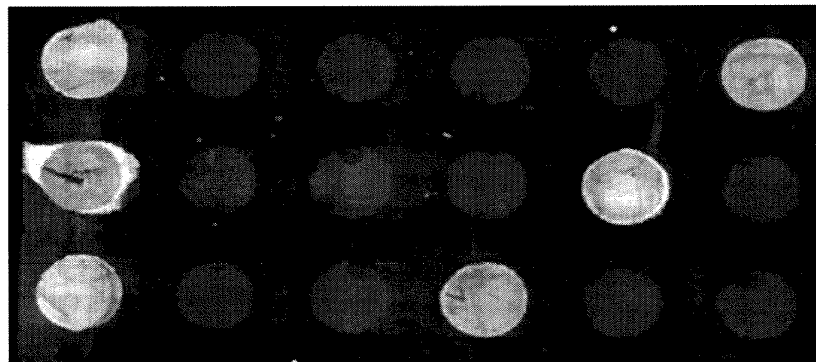


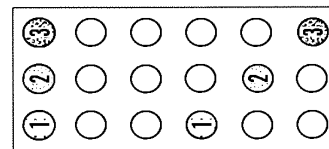
FIG. 7

STEP 2

STEP 1 AS OBSERVED



STEP 1
TARGET



STEP 2
TARGET

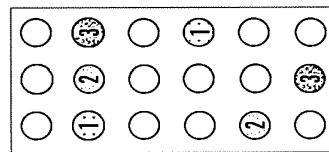
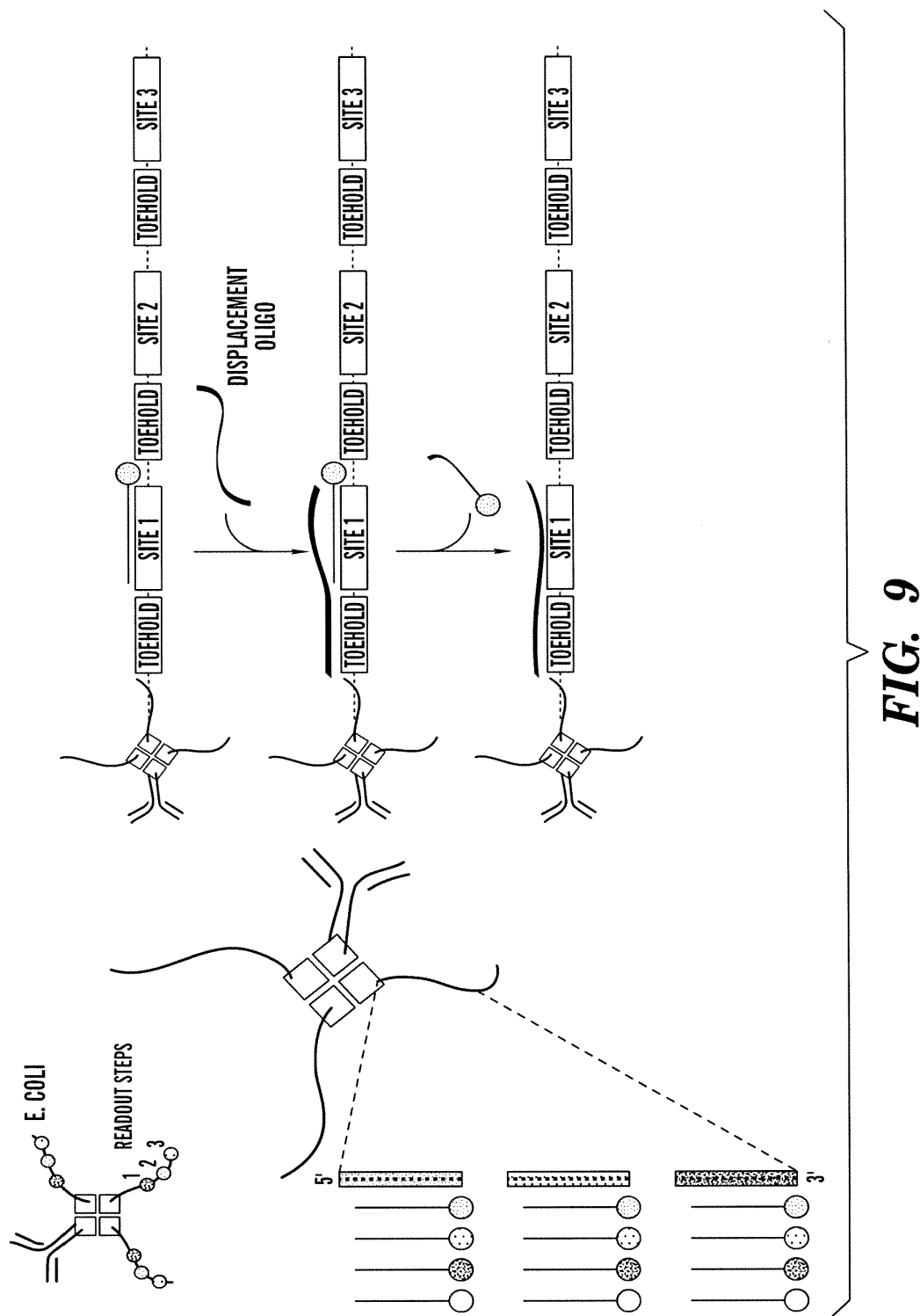


FIG. 8

SUBSTITUTE SHEET (RULE 26)



SUBSTITUTE SHEET (RULE 26)

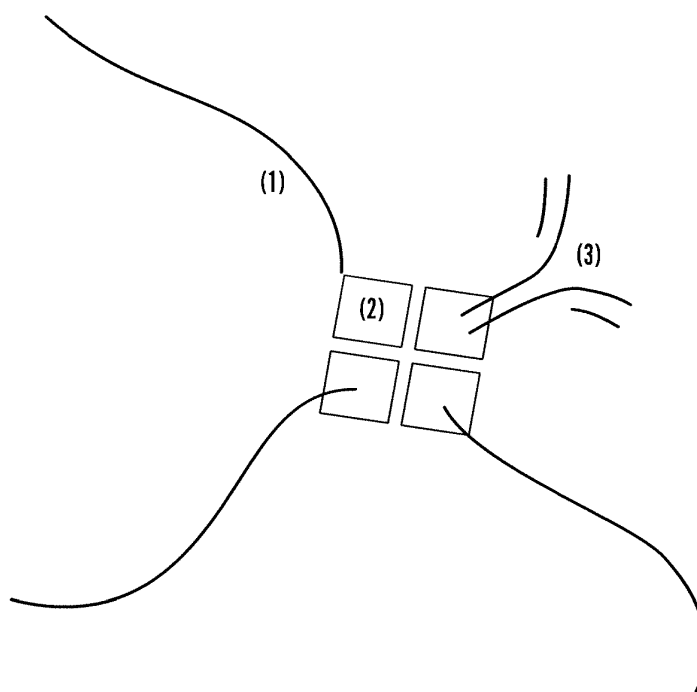


FIG. 10

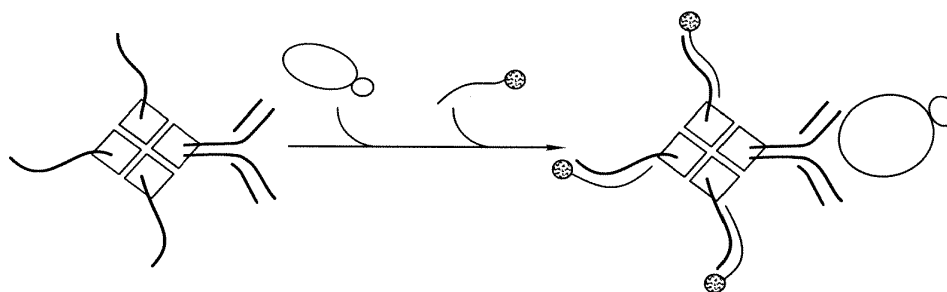


FIG. 11

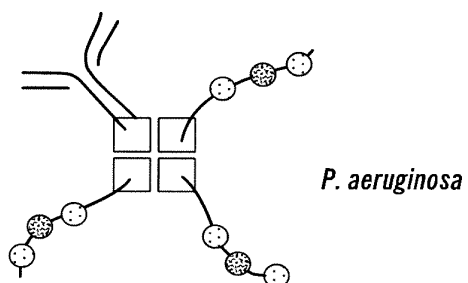
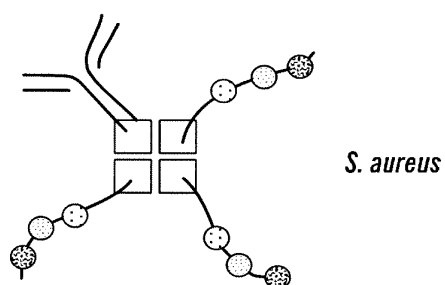
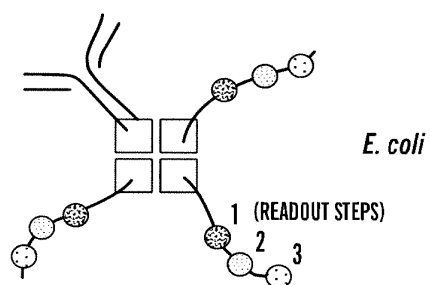
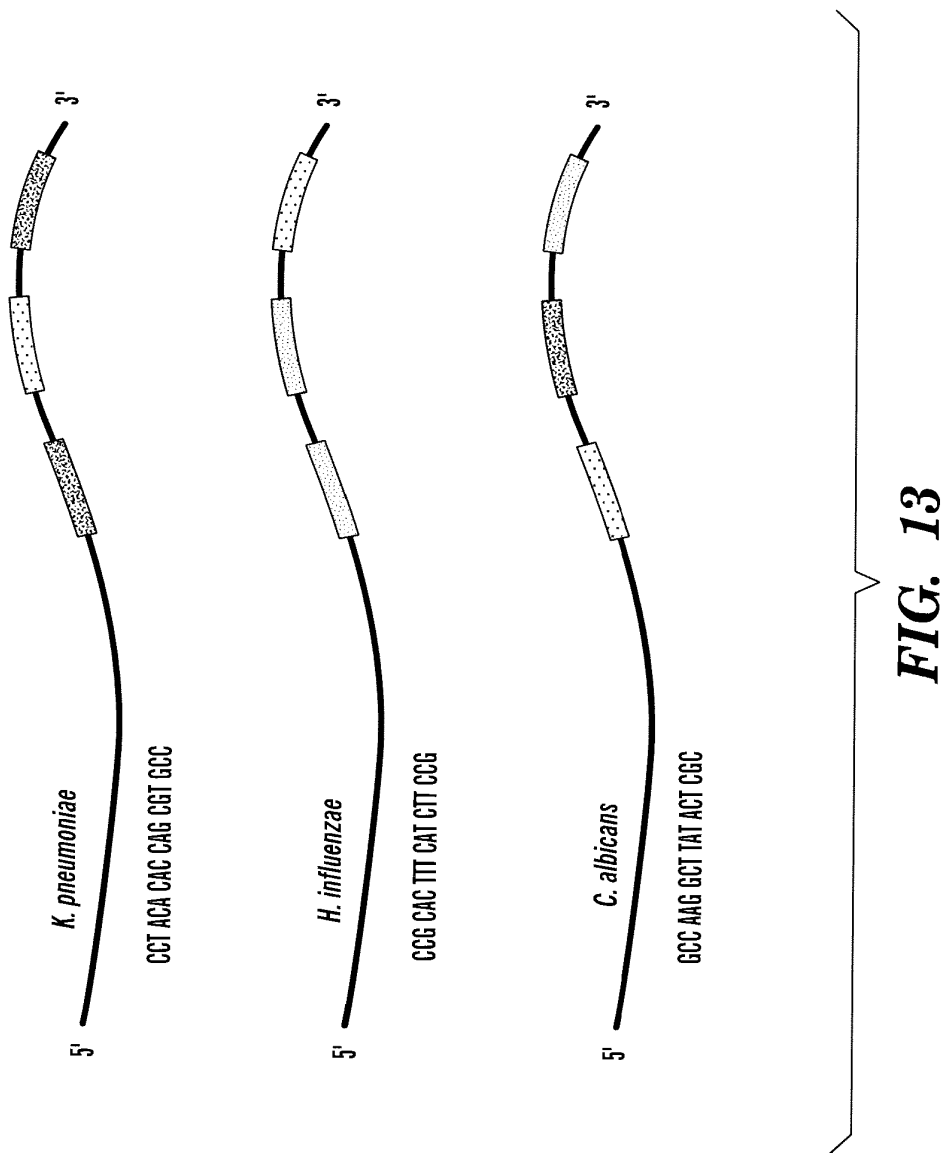


FIG. 12



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/071398**A. CLASSIFICATION OF SUBJECT MATTER***C12Q 1/68(2006.01)i, C12N 15/11(2006.01)i, G01N 33/68(2006.01)i, G01N 33/532(2006.01)i*

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q 1/68; C07K 19/00; G01N 33/48; G01N 33/53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models
Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & Keywords:temporal, plural, detection, analyte, reagent, nucleic acid, label, conjugate, signal, optical

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2011-0294135 A1 (CARLSON) 01 December 2011 See claims 1, 10, 13 and 24; paragraphs 0067, 0109, 0115 and 0119; and figure 1.	1-3,70-73
A	BRENNER et al., 'Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays', Nature Biotechnology, Vol. 18, pp. 630-634 (31 June 2000) See figure 2.	1-3,70-73
A	US 2009-0208965 A1 (TAFAS et al.) 20 August 2009 See the whole document.	1-3,70-73
A	KR 10-2008-0003402 A (MYCOSOL, INC.) 07 January 2008 See the whole document.	1-3,70-73
A	HAN et al., 'Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules', Nature Biotechnology, Vol. 19, pp. 631-635 (31 July 2001) See the whole document.	1-3,70-73



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

05 April 2013 (05.04.2013)

Date of mailing of the international search report

08 April 2013 (08.04.2013)

Name and mailing address of the ISA/KR


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Telephone No. 82-42-481-3371



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2012/071398**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: See extra sheet.
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
See extra sheet.
3. ☒ Claims Nos.: See extra sheet.
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required 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.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2012/071398

(Continuation of Box No.II)

2. Claims Nos.: 9, 12, 15, 23-24, 31, 36, 38, 40-41, 45, 47, 54-55, 59, 61, 75, 77, 82, 84, 92, 97, 99, 109 and 114

Claims 9, 12, 15, 23-24, 31, 36, 38, 40-41, 45, 47, 54-55, 59, 61, 75, 77, 82, 84, 92, 97, 99, 109 and 114 do not comply with PCT Article 6 because they are referring to multiple dependent claims which do not comply with PCT Rule 6.4(a).

3. Claims Nos.: 4-8, 10-11, 13-14, 16-22, 25-30, 32-35, 37, 39, 42-44, 46, 48-53, 56-58, 60, 62-69, 74, 76, 78-81, 83, 85-91, 93-96, 98, 100-108, 110-113 and 115-117

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US2012/071398

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2011-0294135 A1	01.12.2011	EP 2185725 A1 EP 2185725 A4 WO 2009-018576 A1	19.05.2010 12.01.2011 05.02.2009
US 2009-0208965 A1	20.08.2009	AU 2007-329748 A1 CA 2667517 A1 CN 101754973 A EP 2078030 A2 EP 2406390 A1 JP 2010-508028 A KR 10-2009-0071667 A US 2008-0182253 A1 US 2008-0213769 A1 WO 2008-070333 A2 WO 2010-104533 A2	12.06.2008 12.06.2008 23.06.2010 15.07.2009 18.01.2012 18.03.2010 01.07.2009 31.07.2008 04.09.2008 12.06.2008 16.09.2010
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(25) Filing Language: English

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61/594,480 3 February 2012 (03.02.2012) US
61/703,093 19 September 2012 (19.09.2012) US

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

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SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS**CROSS-REFERENCE**

[0001] This application claims the benefit of U.S. provisional applications 61/594,480, filed February 3, 2012 and 61/703,093, filed September 19, 2012, each of which is incorporated herein by reference in its entirety.

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 (c.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	
	~~CC~~	~~1~~	~~1~~	~~0~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	E	1	1	0	1	
	~~EE~~	~~1~~	~~1~~	~~0~~	~~1~~	Eliminated from coding scheme to eliminate degeneracy.
	G	1	1	1	1	
<i>Cumulative Maximum Result</i>		6	3	2	3	
2	H	1	0	0	4	
	I	1	0	3	0	
	J	1	4	0	0	
	~~HH~~	~~1~~	~~0~~	~~3~~	~~4~~	Eliminated from coding scheme to eliminate degeneracy.
	L	1	4	0	4	
	~~LL~~	~~1~~	~~4~~	~~3~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	N	1	4	3	4	
<i>Cumulative Maximum Result</i>		11	15	8	15	
3	O	1	0	0	16	
	P	1	0	9	0	
	Q	1	16	0	0	
	~~OO~~	~~1~~	~~0~~	~~9~~	~~16~~	Eliminated from coding scheme to eliminate degeneracy.
	S	1	16	0	16	
	~~SS~~	~~1~~	~~16~~	~~9~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	U	1	16	9	16	
<i>Cumulative Maximum Result</i>						
<i>Cumulative Maximum Result for Three Non-Degenerate Tiers</i>		16	63	26	63	

[0099] The right-hand side of **Fig. 1** shows one exemplary embodiment of the method described above. More specifically, the right-hand side of **Fig. 1** shows an encoding method where nine or more analytes (Seq 1 - Seq 9, etc.) are each encoded by at least two colors, with varying intensities within each color. The color represents a fluorophore attached to an oligonucleotide probe that also comprises a quencher. The system can be designed so that each probe is labeled with single

fluorophore, or each probe is labeled with more than one fluorophore. For example, the code for analyte H, in Table 6, is 1004. The intensity of 4 in the red channel may be achieved by either using an H-specific probe comprising 4 red fluorophores, or by using 4 H-specific probes each comprising a single red fluorophore. Of course, any combination of probes and fluorophores producing a result of 4 in the red channel would be equally appropriate, such as 2 probes with 2 red fluorophores each, and 1 probe with 1 red fluorophore and 1 probe with 3 red fluorophores, or simply one probe with one red fluorophore but present at 4x amount in the reaction mixture.

[00100] The coding scheme depicted on the right-hand side of **Fig. 1** may be represented as in Table 7. The result of the analysis shown on the right-hand side of **Fig. 1** is 4112, or an intensity of 4 in the blue channel, 1 in the green channel, 1 in the yellow channel, and 2 in the red channel. Using a decoding matrix constructed as described herein, this result is decoded to indicate the presence of Seq 1, Seq 3, Seq 4, and Seq 5.

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

Tier	Analyte	B	G	Y	R	Comments
1	Seq 1	1	0	0	0	
	Seq 2	1	1	0	0	
	Seq 3	1	0	1	0	
	Seq 4	1	0	0	1	
	Seq 5	1	1	0	1	
	Seq 6	1	1	1	1	
	Eliminated Potential Seq	1	0	1	1	Eliminated from coding scheme to eliminate degeneracy.
	Eliminated Potential Seq	1	1	1	0	Eliminated from coding scheme to eliminate degeneracy.
Cumulative Maximum Result		6	3	2	3	
2	Seq 7	1	4	0	0	
	Seq 8	1	0	3	0	
	Seq 9	1	0	0	4	
Etc. - i.e. Continue as provided in Table 6.						

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

[00101] In some methods provided herein, each analyte is encoded by a single color and intensity combination. For example, in a four color system, the first four analytes may be encoded

by 1000, 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 genres *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 parvaptensis*, *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 genres *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 microti*, *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 bovis*, *Sarcocystis suis*, *Schistosoma mansoni*, *Toxoplasma gondii*, *Trichomonas vaginalis*, *Trypanosoma brucei*, and *Trypanosoma cruzi*.

[00113] Examples of fungi include those from the genres *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,

thiacarbocyanine, merocyanine, cyanine 2, cyanine 3, cyanine 3.5, cyanine 5, cyanine 5.5, cyanine 7, oxadiazole derivatives, pyridyloxazole, nitrobenzoxadiazole, benzoxadiazole, pyrene derivatives, cascade blue, oxazine derivatives, Nile red, Nile blue, cresyl violet, oxazine 170, acridine derivatives, proflavin, acridine orange, acridine yellow, arylmethine derivatives, auramine, crystal violet, malachite green, tetrapyrrole derivatives, porphin, phtalocyanine, and bilirubin. Exemplary quenchers include black hole quenchers, such as BHQ-0, BHQ-1, BHQ-2, BHQ-3; ATTO quenchers, such as ATTO 540Q, ATTO580Q, and ATTO612Q; and the like.

[00151] The fluorophores that may be used with the invention are not limited to any of the fluorophores described herein. For example, fluorophores with improved properties are continually developed, and these fluorophores could readily be used with the methods provided in this disclosure. Such improved fluorophores include quantum dots, which may emit energy at different wavelengths after being excited at a single wavelength. The advantage of using such fluorophores is that only a single excitation source is needed, but many different signals may be quantified, for example in terms of color and intensity. Moreover, fluorophores with narrow emission spectra would be particularly useful with the method described herein, as such fluorophores could be included in multiplex assays with minimal or no overlap between their emission spectra, thereby offering many more “colors” and boosting significantly the overall number of coded analytes.

[00152] In some cases, one or more reagents are lyophilized. Any suitable reagent may be lyophilized. For example, probes, primers, enzymes, antibodies, or any other reagent used for detection may be lyophilized. In some cases, a sample comprising an analyte may also be lyophilized. Lyophilization may be useful, for example, when distributing reagents and/or samples in developing 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 GTTTGCCAGGAAGATGGAACCAAAAATGATAGGGGGAATTGGAGGTTTT ATCAAAGTAAGACAGTATGATCAGATACTCATAGAAATCTGTGGACATAA AGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGG
Forward Primer (3' to 5')	GGAAGCTCTATTAGATACAGGAGCAG

Reverse Primer (3' to 5')	CCAATTATGTTGACAGGTGTAGGTCC
Probe 1 (3' to 5')	/56-FAM/TGAGTTTGCCAGGAAGATGGAAACCA/3BHQ_1/

Table 10. HIV p17 sequence information.

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

Table 11. *Plasmodium* Chr7 sequence information.

Sequence Name	<i>Plasmodium</i> Chr7 199mer synthesized from bases 1139138 - 1141223
Source	UCSC <i>Plasmodium falciparum</i> Genome Browser Gateway
Target sequence (3' to 5')	GCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAG AGGGAGCCTGAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTA AATTACCCAATTCTAAAGAAGAGAGGTAGTGACAAGAAATAACAATGCAA GGCCAATTTAAACCTTCCCAGAGTAACAATTGGAGGGCAAGTCTGGTG
Forward Primer (3' to 5')	GCCTAACATGGCTATGACGGGTAA
Reverse Primer (3' to 5')	CACCAGACTTGCCCTCCAATTGTT
Probe 1 (3' to 5')	/56-FAM/ATTCCGGAGAGGGAGCCTGAGAAATA/3BHQ_1/
Probe 2 (3' to 5')	/56-ROXN/AAGGAAGGCAGCAGGCGCGTAAATTA/3BHQ_2/

Table 12. Herpes simplex virus 2 sequence information.

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

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

Table 13. *Mycobacterium tuberculosis* rpoB sequence information

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

Table 14. Dengue virus type 3 sequence information.

Sequence Name	Dengue Virus Type 3 200mer synthesized from Dengue Virus Type 3 genome
Source	GenBank M93130
Target sequence (3' to 5')	ATGCCAACTGTGATTGAGCACTTAGAAAGACTACAAAGGAAACATGGAGG AATGCTTGTGAGAAATCCACTCTCACGAACTCCACGCACGAAATGTATT GGATATCCAATGGTACAGGCAACATCGTCTCATTACAATGACACACAGG AGACCCACCATAGAGAAAGATGTGGATTTAGGAGCAGGAACCCGACATGT
Forward Primer (3' to 5')	ATGCCAACTGTGATTGAGCACT

Reverse Primer (3' to 5')	ACATGTCGGGTTCTGCTCCTAAA
Probe 1 (3' to 5')	/56-FAM/ACAAAGGAAACATGGAGGAATGCTTGTGA/3BHQ_1/
Probe 2 (3' to 5')	/5Cy3/ACTCTCACGAAACTCCACGCACGAAA/3BHQ_2/
Probe 3 (3' to 5')	/56-ROXN/ACAATGACACACAGGAGACCCACCAT/3BHQ_2/
Probe 4 (3' to 5')	/5Cy5/GGATATCCAATGGTACAGGCAACATCGT/3IAbRQSp/

3. Polymerase Chain Reactions

[00175] All PCR reactions were performed in a ROCHE 480 LIGHTCYCLER instrument. Forty-five thermal cycles were performed, with a 60 second hot-start at 95°C. The cycling conditions for denaturation, annealing, and extension were 45 seconds, 50 seconds, and 60 seconds at 95°C, 65°C, and 70°C, respectively. Each experiment was run in quintuplicate, with a reaction volume of 15 µL. Fluorescence measurements were obtained in the following channels after every annealing step: 483 nm to 533 nm (FAM), 523 nm to 568 nm (Cy3), 558 nm to 610 nm (ROX), and 615 nm to 670 nm (Cy5). Fluorescence measurements were also obtained after the hot-start and the end of the 45 thermal cycles. The change in the fluorescence intensity between these two measurements (after hot-start and at the end of the 45 cycles) determined the end-point fluorescent signal.

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

[00176] This example describes the simultaneous detection of up to four polynucleotides selected from HIV (2 polynucleotides), HSV-2, *Mycobacterium*, *Plasmodium*, and dengue virus. Fourteen PCR reactions were assembled, using the reagents described in Table 15.

Table 15. Reagents for 14 PCR reactions.

Reaction No.	Type of Reagent	Reagent	Concentration	Volume
1		UltraPure Water	-	34 µL
		Taq 5x Master Mix	-	42 µL
	Template	Poly Protease Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 nM	2 µL
		Protease RVS Primer	1 nM	2 µL
	Probe	Poly Protease Probe 1	1 nM	2 µL
2		UltraPure Water	-	28 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Malaria Template	15 nM	2 µL

		Herpes Template	17 nM	2 μ L
		TB Template	16 nM	2 μ L
		Dengue Virus Template	17 nM	2 μ L
	<i>Primers</i>	Poly Protease FWD Primer	1 nM	2 μ L
		Protease RVS Primer	1 nM	2 μ L
	<i>Probe</i>	Poly Protease Probe 1	1 nM	2 μ L
3		UltraPure Water	-	32 μ L
		Taq 5x Master Mix	-	42 μ L
	<i>Template</i>	Malaria Template	15 nM	2 μ L
	<i>Primers</i>	Malaria FWD Primer	1 μ M	2 μ L
		Malaria RVS Primer	1 μ M	2 μ L
	<i>Probes</i>	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
	<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
		Dengue Virus Template	17 nM	2 μ L
	<i>Primers</i>	Malaria FWD Primer	1 μ M	2 μ L
		Malaria RVS Primer	1 μ M	2 μ L
	<i>Probes</i>	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
	<i>Template</i>	Herpes 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
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

	<i>Templates</i>	Taq 5x Master Mix	-	42 μ L
		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
11		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>	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 polypeptase, *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	-	50 μ L

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	1 μ M	4 μ L
Poly Protease RVS Primer	1 μ M	4 μ L
P17 FWD Primer	1 μ M	4 μ L
P17 RVS Primer	1 μ M	4 μ L
Dengue Virus FWD Primer	1 μ M	4 μ L
Dengue Virus RVS Primer	1 μ M	4 μ L
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4 μ L
P17 Probe 1	400nM	4 μ L
Dengue Virus Probe 1	200nM	4 μ L

Table 22. Binary FAM Experiment 5 Cocktail: dengue virus

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

Table 23. Binary FAM Experiment 6 Cocktail: HIV p17

Reagents	Concentration	Volume Added
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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 polypotease

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	4 μ L
P17 Probe 1	400nM	4 μ L
Dengue Virus Probe 1	200nM	4 μ L

[00186] The total fluorescence signal of each experiment was plotted on a chromatogram (**Fig. 7**) that was constructed as described above. The width of the bands around each positive control measurement is the propagated uncertainty of the 1X measurement. That uncertainty was equated to the standard deviation of the fluorescence signals of the last five PCR cycles in saturation.

[00187] **Fig. 7** shows the results of this experiment. The three right-most results show the measured intensities for dengue virus alone (DEN), HIV p17 alone (P17), and HIV polyprotease (TPP). The assigned intensities for each of these analytes were as indicated in Table 11, and **Fig. 7** confirms that the three analytes yielded the expected intensity results when alone (see DEN, P17, and TPP). At the end of the assay, essentially all of the probes have been hydrolyzed, releasing essentially all of the fluorophores, which emit a signal. A fixed amount of a fluorophore produces a fixed and predictable amount of signal. Therefore a target that is present should generally produce a fixed amount of signal, as determined by the amount of probe added to the reaction volume.

[00188] As expected, the intensity values for the combinations of analytes are equivalent to the sum of the assigned intensities. Since this coding scheme is designed to be non-degenerate, each result can be unambiguously decoded into the presence or absence of a particular analyte. For example, starting from the left side of **Fig. 7**, a result of 7 indicates the presence of all three analytes. A result of 3 indicates the presence of dengue virus and HIV p17 analytes, and the absence of HIV polyprotease. A result of 5 indicates the presence of dengue virus and HIV polyprotease, and the absence of HIV p17. Finally a result of 6 indicates the presence of HIV p17 and HIV polyprotease, and the absence of dengue virus. This coding scheme can be extended to encode additional analytes, as described elsewhere in this disclosure.

CLAIMS**WHAT IS CLAIMED IS:**

1. 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 analysis.
2. The assay of claim 1, wherein each of said analytes is encoded as a value of one component of a signal.
3. The method of claim 2, wherein said method 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 said one component of said signal when all of said analytes are present.
4. The assay of claim 1, wherein each of said analytes is encoded as at least one first value in a first component of said signal and at least one second value in a second component of said signal.
5. The assay of claim 4, wherein each of said analytes is encoded as a first value in a first component of said signal at each of a plurality of second values in a second component of said signal.
6. The method of claim 4, wherein said method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = C * \log_2 (F + 1)$, where C is the number of said second values used to encode said analytes and F is the maximum cumulative value of said first component of said signal, for any second value, when all of said analytes are present.
7. The method of claim 4, wherein said method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = (P * T) + 1$, where P is the number of codes per tier in a coding scheme and T is the number of tiers.
8. The method of claim 7, wherein the number of tiers $T = \log_4 (F + 1)$, and F is the maximum cumulative value of said first component of said signal, for any second value, when all of said analytes are present.
9. The method of claim 4, wherein said first value is an intensity or range of intensities.
10. The method of claim 9, wherein said coding scheme comprises at least three intensities or ranges of intensities.
11. The method of claim 4, wherein said second value is a wavelength or range of wavelengths.
12. The method of claim 11, wherein said coding scheme comprises at least five wavelengths or ranges of wavelengths.

13. The method of claim 1, wherein said signal is an electromagnetic signal.
14. The method of claim 13, wherein said electromagnetic signal is a fluorescence emission signal.
15. The method of claim 14, wherein intensity of said fluorescence emission signal is measured at at least four wavelengths or ranges of wavelengths.
16. The method of claim 1, wherein said detecting is performed with reagents that are lyophilized prior to use.
17. The method of claim 1, wherein said detecting is performed with reagents comprising hybridization probes.
18. The method of claim 17, wherein the number of said hybridization probes is greater than the number of said analytes.
19. The method of claim 17, wherein said hybridization probes comprise one or more hybridization probes specific for different analytes and comprising an identical fluorophore or combination of fluorophores.
20. The method of claim 17, wherein said sample is contacted with at least 18 of said hybridization probes.
21. The method of claim 17, wherein said reagents further comprise at least one pair of primers capable of amplifying a region complementary to at least three of said hybridization probes.
22. The method of claim 21, wherein said signal is generated during a polymerase chain reaction.
23. The method of claim 22, wherein said polymerase chain reaction is selected from the group consisting of an end-point polymerase chain reaction, a real-time polymerase chain reaction, and a digital polymerase chain reaction.
24. The method of claim 1, wherein said cumulative measurement is performed on a solution.
25. The method of claim 1, 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.
26. The method of claim 25, wherein said at least one additional value is 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.

27. The method of claim 1, further comprising constructing a chromatogram.
28. The method of claim 27, wherein said chromatogram is constructed by plotting all possible combinations of said first values and said second values for positive control samples.
29. The method of claim 1, wherein at least one of said analytes comprises a polynucleotide.
30. The method of claim 29, wherein said polynucleotide is from a source selected from the group consisting of an animal, a plant, a bacteria, a fungus, a parasite, and a virus.
31. The method of claim 23, wherein said 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.
32. The method of claim 31, wherein said 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.
33. The method of claim 1, wherein said 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.
34. The method of claim 1, further comprising transmitting information concerning the presence or absence of said analyte through a computer network.
35. The method of claim 1, further comprising providing information concerning the presence or absence of said analyte to a physician.
36. The method of claim 35, further comprising making a clinical decision based on said information.
37. The method of claim 1, wherein at least one step of said method is performed using instructions on a computer readable medium.
38. The method of claim 37, wherein said instructions are located on a remote server.
39. The method of claim 37, wherein said instructions are located on a thermal cycler.
40. The method of claim 37, wherein said instructions are located on a computer in communication with a thermal cycler.
41. The method of claim 1, wherein at least one of said analytes is a positive control analyte.
42. The method of claim 1, wherein said coding scheme is non-degenerate.
43. The method of claim 42, wherein said coding scheme is designed to be non-degenerate.

44. The method of claim 42, 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.
45. The method of claim 1, wherein said assay is an end-point assay.
46. The method of claim 1, wherein said assay is ended at a threshold number of cycles set by the limit of detection of an instrument.
47. The method of claim 1, wherein said assay is a liquid phase assay.
48. The method of claim 1, wherein said assay is quantitative.
49. 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.
50. The method of claim 49, wherein said method 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 said first values when all of said analytes are present.
51. The method of claim 49, wherein said first value is an intensity or range of intensities.
52. The method of claim 49, wherein said first value has a minimum value in said 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.
53. The method of claim 49, wherein said first value is incremented in said coding scheme by an amount equal to the cumulative maximum of said preceding first values plus one.
54. The method of claim 49, wherein said first value is incremented in said coding scheme by an amount greater than the cumulative maximum of said preceding first values plus one.

55. 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.
56. The method of claim 55, wherein each of said analytes is encoded as a first value in a first component of said signal at each of a plurality of second values in a second component of said signal.
57. The method of claim 55, wherein, when degeneracy is eliminated, said 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.
58. The method of claim 55, wherein, when degeneracy is eliminated, said coding scheme comprises T non-degenerate tiers, wherein $T = \log_4 (F + 1)$ and F is the maximum cumulative value of said first component of said signal, for any second value, when all of said analytes are present.
59. The method of claim 56, wherein, when degeneracy is eliminated, said 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.

60. The method of claim 55, wherein, when degeneracy is eliminated, said 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 said coding scheme, and F is the maximum cumulative value of said first component of said signal, for any second value, when all of said analytes are present.
61. 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.
62. The non-degenerate coding scheme of claim 61, wherein said coding scheme is generated by a method comprising:
- 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;
 - enumerating every legitimate cumulative result for all possible combinations of presence or absence of each analyte;
 - identifying each legitimate result that is degenerate; and
 - eliminating at least one code to eliminate degeneracy.
63. The method of claim 62, further comprising expanding said coding scheme by adding additional codes that are at least one unit greater than the sum of all previous codes in said at least one component of said signal.
64. The method of claim 61, wherein said coding scheme is generated by a method of mathematical iteration that guarantees non-degeneracy by construction.
65. 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.

66. 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.

67. 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.
68. 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;
 - i. 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
 - l. providing said party with information about the presence or absence of each of said analytes.
69. 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.

70. 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.
71. 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.
72. 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.
73. 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.
74. 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.

Fig. 1

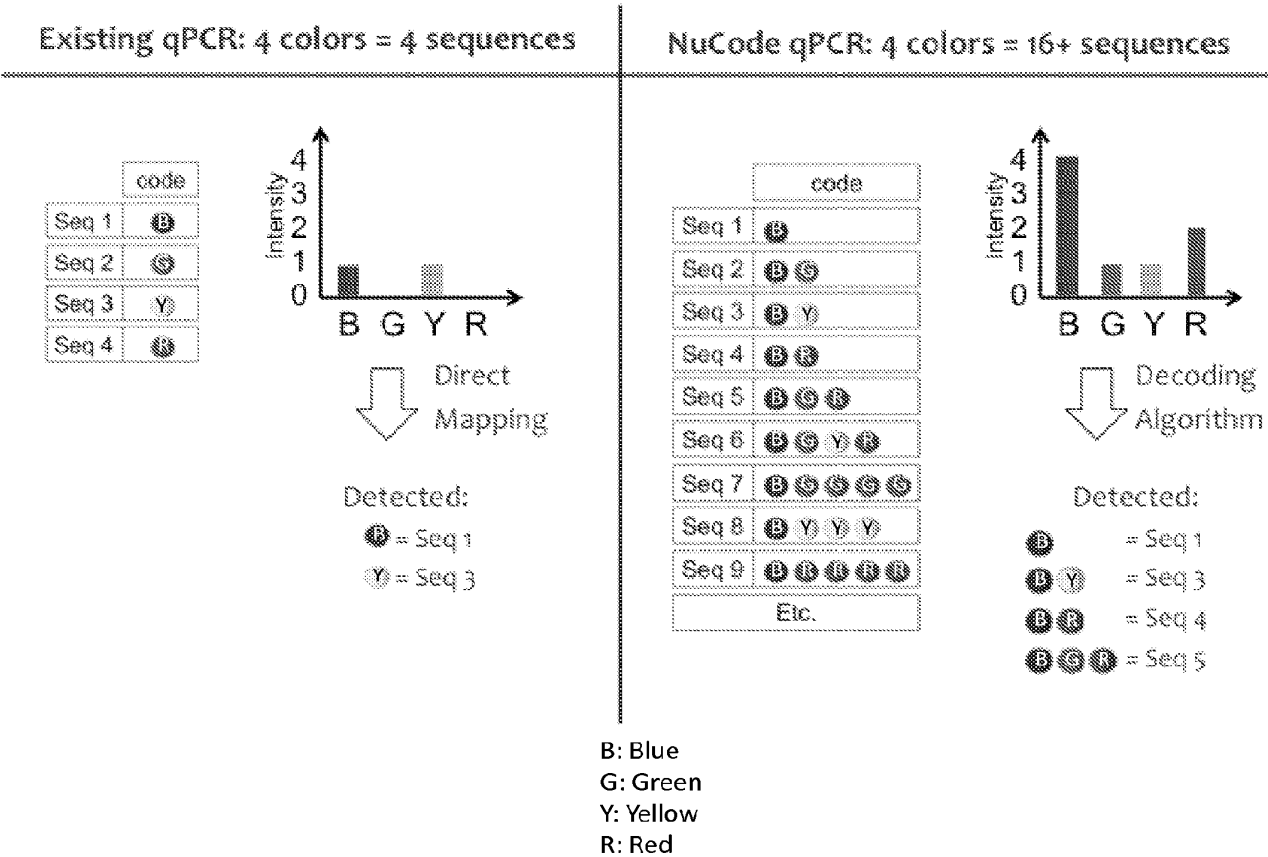


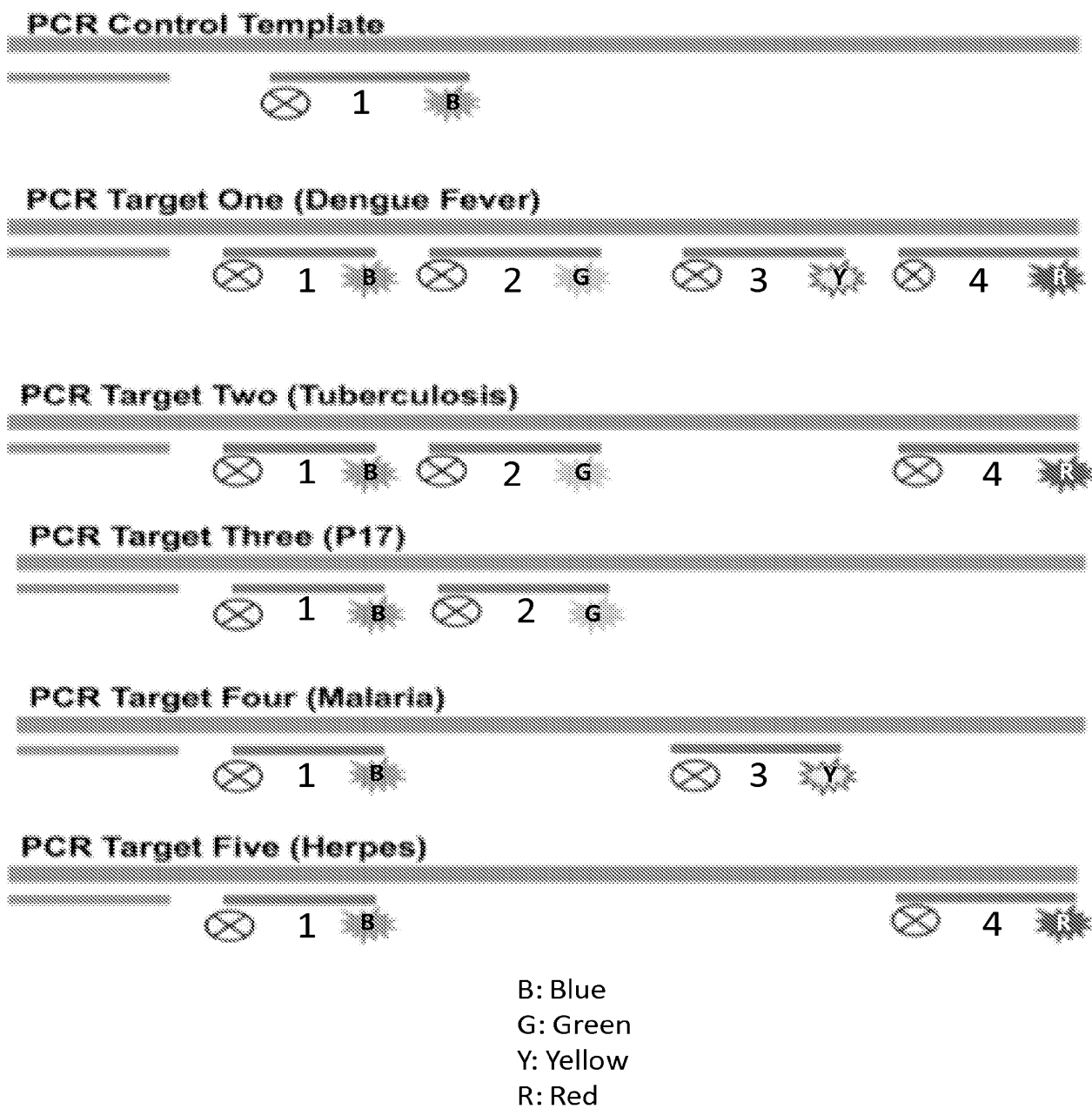
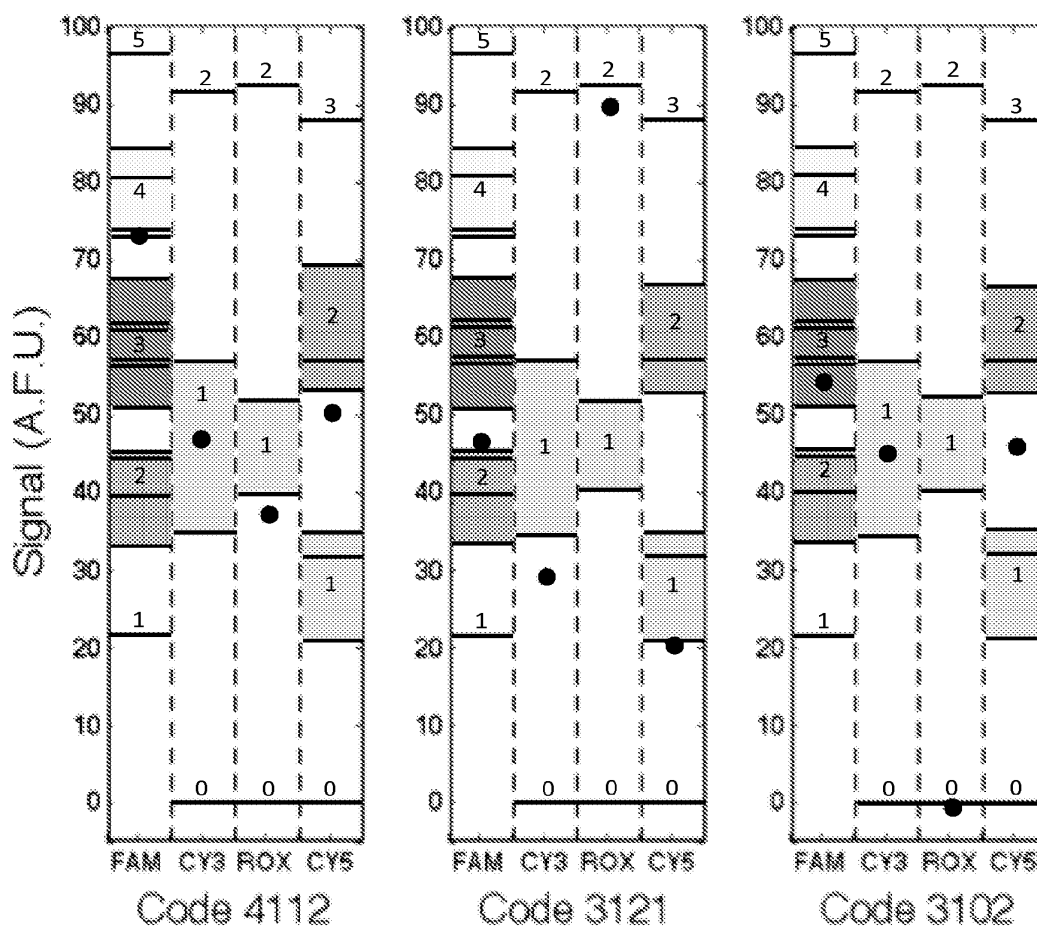
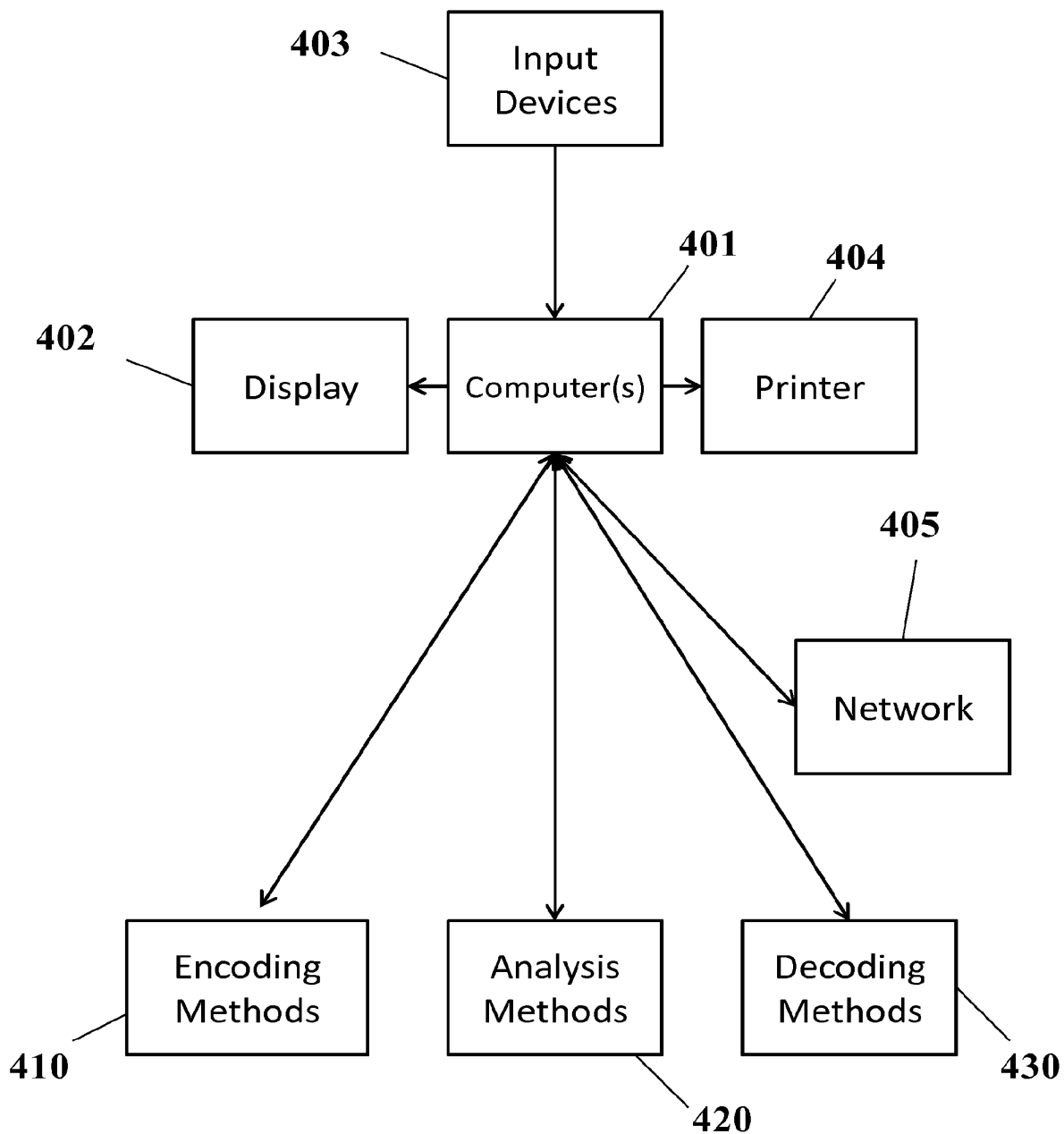
Fig. 2

Fig. 3

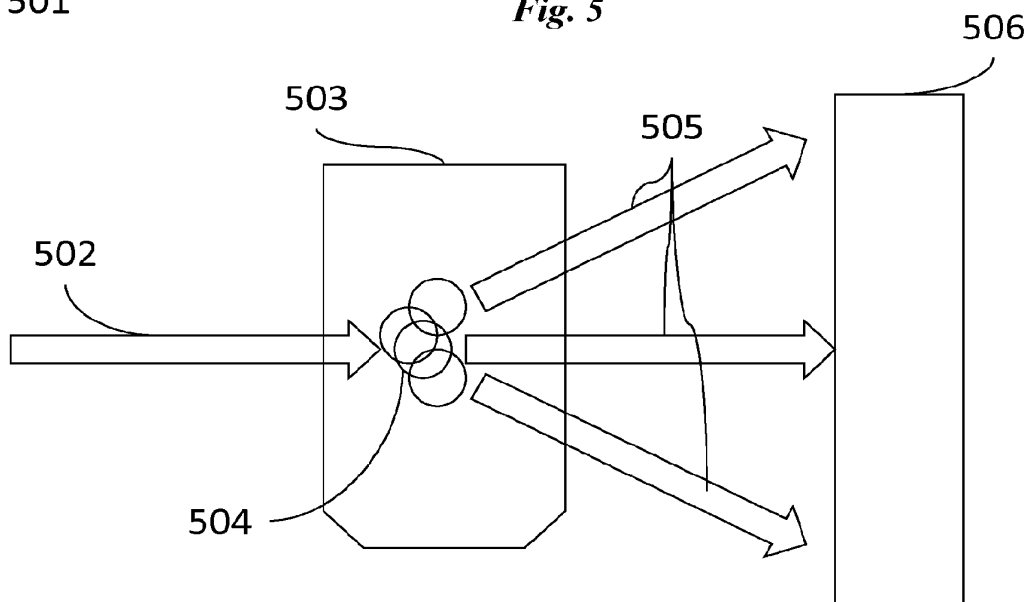
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Fig. 4

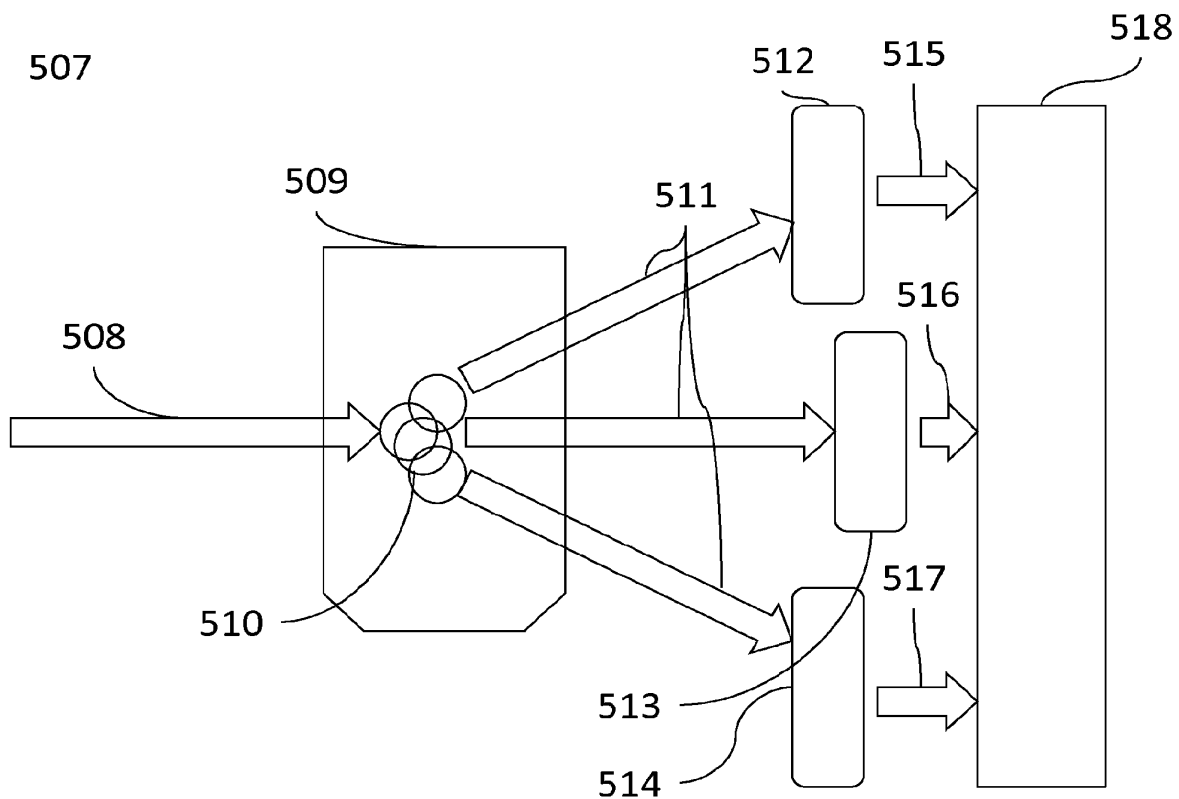
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501

Fig. 5



507



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Fig. 6

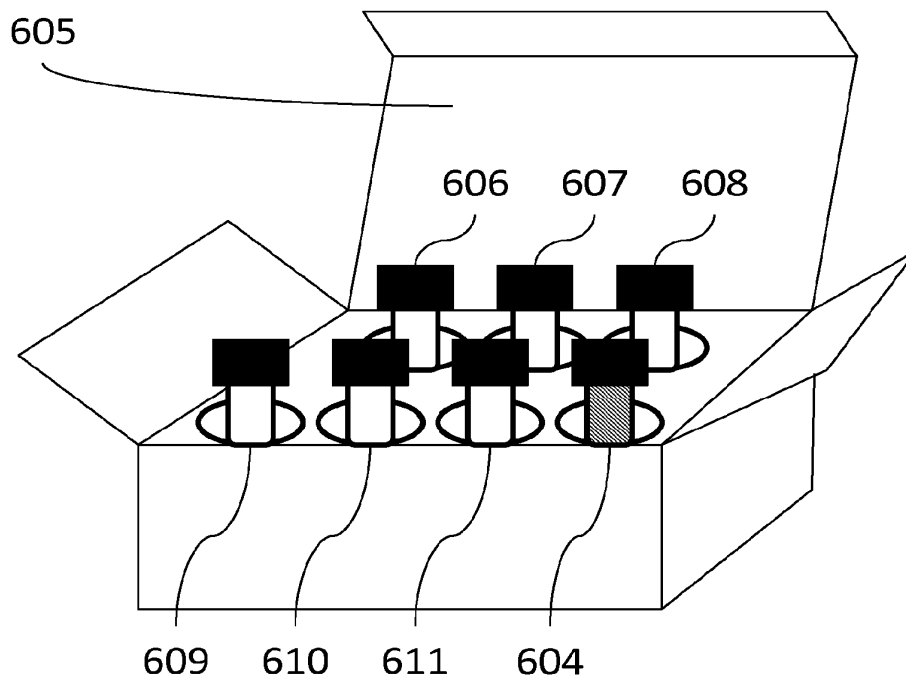
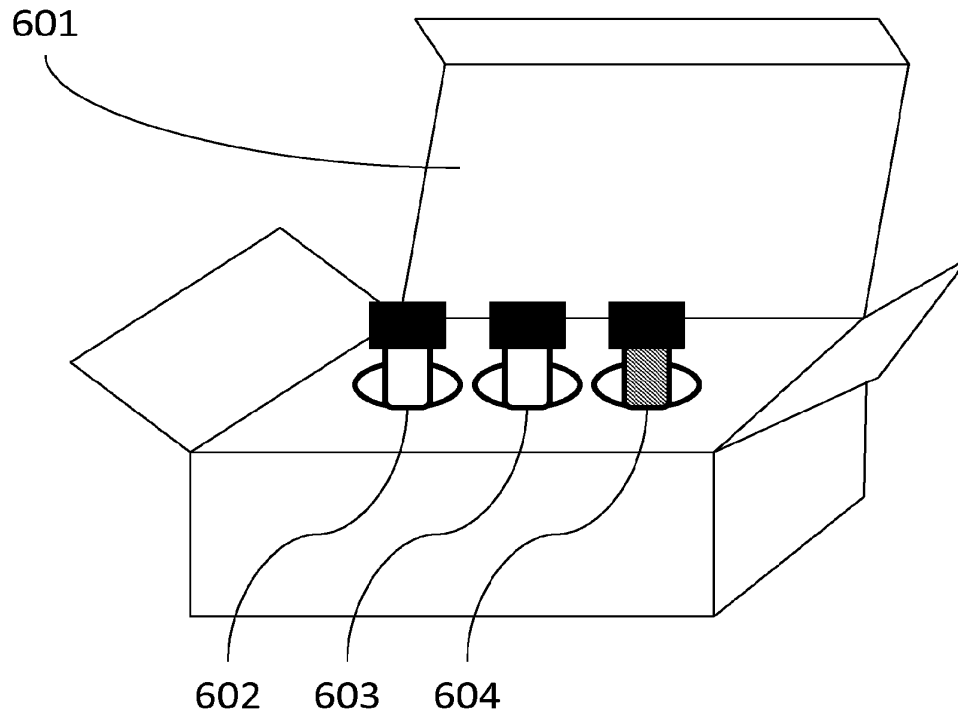


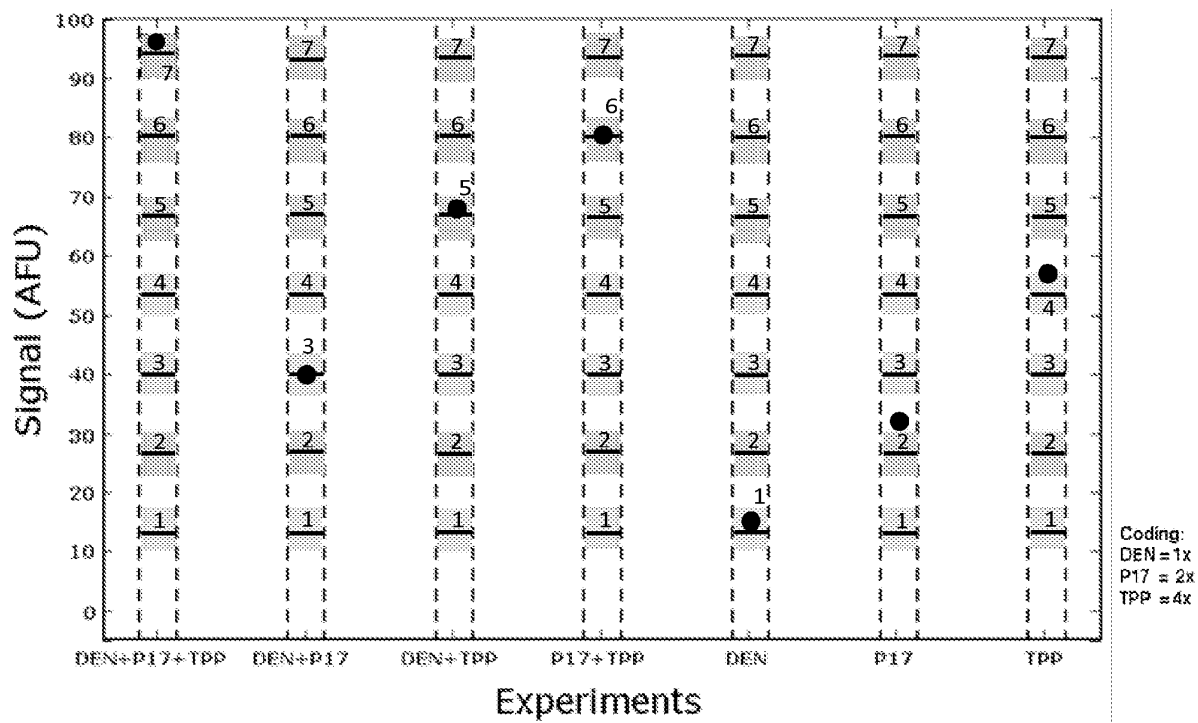
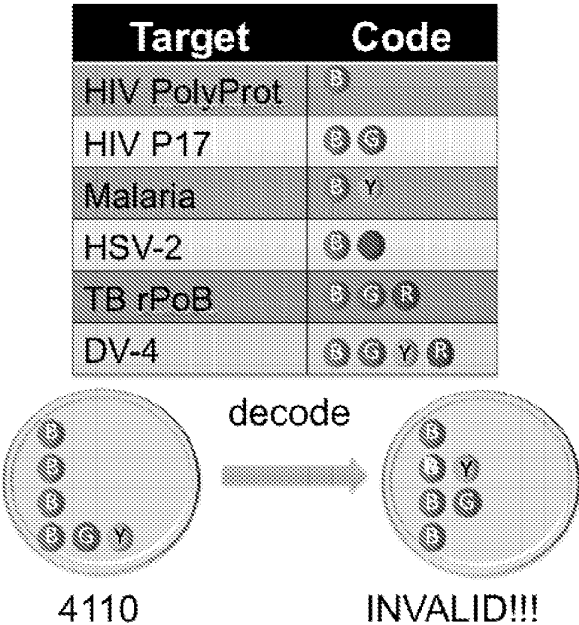
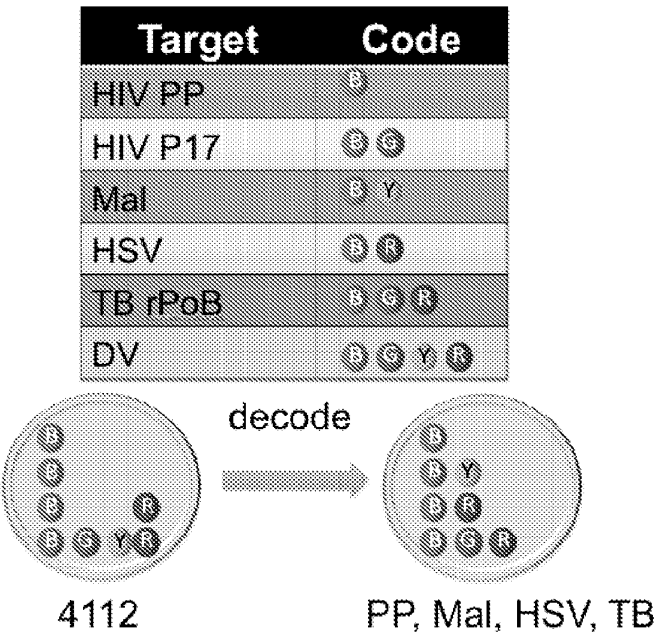
Fig. 7

Fig. 8



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
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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

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[Continued on next page]

(54) Title: MULTIPLEXING AND QUANTIFICATION IN PCR WITH REDUCED HARDWARE AND REQUIREMENTS

(57) Abstract: Methods and algorithms for a multiplexed single detection channel amplification process and quantification of generated amplicons is presented. Various mathematical approaches for quantifying and verifying the amplicons in a reaction are presented. Usage of such methods and approaches allow upgrading of existing single and multiple channel instruments for further multiplexing capabilities.

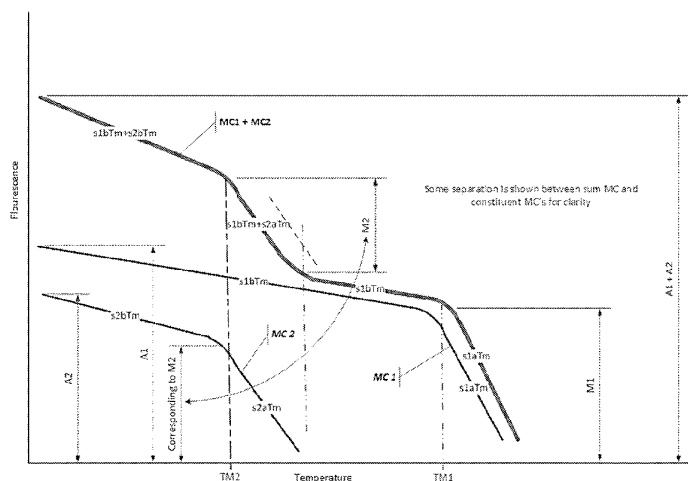


FIG. 3

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Multiplexing and Quantification in PCR with Reduced Hardware and Requirements**CROSS REFERENCE TO RELATED APPLICATIONS**

[001] The present application claims priority to US provisional application No. 61/679,547 for “Multiplexing and Quantification in PCR with Reduced Hardware and Requirements”, filed on Aug. 3rd, 2012, which is herein incorporated by reference in its entirety.

TECHNICAL FIELD

[002] The present disclosure relates to the field of biochemical technology, and more specifically to methods and algorithms used in a multiplexed real-time quantitative polymerase chain reaction (qPCR) technology.

BACKGROUND

[003] Multiplexing in real-time quantitative polymerase chain reaction (qPCR) allows detection and quantification of different amplified targets (e.g. amplicons) within a single qPCR assay, thus conserving sample material (e.g. enzyme, nucleotides, etc.) and avoiding well to well variation which can occur if multiplexing is done by splitting the sample into multiple separate chambers, well or tubes. Typical implementation of multiplexed qPCR requires reporters with different spectra (e.g. emission wavelengths) for each different amplicon and different detection channels to detect each of the different spectra. In one particular case, special reporters (e.g. target specific probes) are used to allow multiplexed qPCR using a same emission spectrum (e.g. wavelength) thus reducing the hardware required for the detection of the emission (e.g. reduced number of detection channels). Teachings according to the present disclosure allow for multiplexed qPCR and quantification in a single channel detection, by using, for example, simple non-specific dyes (e.g. intercalating dyes) and a same emission spectrum, thus providing a simple and cost effective multiplexing and quantification solution.

SUMMARY

[004] According to a first aspect of the present disclosure, a method is provided, the method comprising: 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; quantifying the plurality of detected different amplicons, and verifying the plurality of detected different amplicons, wherein the quantifying and the verifying comprise using mathematical analysis of the generated sum amplitude signal.

[005] According to a second aspect of the present disclosure, a processor-based hardware analyzer for analyzing a multiplexed amplification reaction is provided, 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.

BRIEF DESCRIPTION OF THE DRAWINGS

[006] The accompanying drawings, which are incorporated into and constitute a part of this specification, illustrate one or more embodiments of the present disclosure and, together with the description of example embodiments, serve to explain the principles and implementations of the disclosure.

FIGs. 1A-1D show the various possible relationships between the amplitude curves of a multiplexed reaction with two amplicons.

FIG. 2 shows a flowchart of an algorithm according to the present disclosure for a single color detection multiplexed reaction.

FIG. 3 shows an exemplary sum melt curve of two amplicons and its two constituent melt curves associated to each of the amplicons.

FIG. 4 shows exemplary amplification curves of two amplicons with threshold cycle (C_T) points close to each other.

FIGs. 5A and 5B show a typical three step and two step respectively temperature cycle of an amplification process and melting temperatures associated to amplified amplicons.

FIG. 6B shows an embodiment according to the present disclosure of a modified temperature cycle of the three step cycle depicted in FIG. 6A, wherein segments of a transition region around various melting temperatures of amplicons are modified to have less steep slopes, the transition region being a ramping down of the temperature of the cycle.

FIGs. 7A and 7B are equivalent to FIGs. 6A/6B for a case of a two step cycle and wherein the transition region is a ramping up of the temperature of the cycle.

FIG. 8 shows a excitation and emission spectra of SYBR Green and Alexa Fluor dyes, together with a wide detection filter used to detect portion of the emission spectra of both dyes.

FIG. 9 shows an estimation model which according to an embodiment of the present disclosure can be used to represent a multiplexed amplification process.

FIG. 10 shows error in minimum cumulative point wise distance between two normalized and two non-normalized curves.

FIG. 11 is an exemplary embodiment of a processor-controlled hardware used in a single color detection multiplexed amplification reaction.

DETAILED DESCRIPTION

[007] According to several embodiments of the present disclosure, methods and algorithms for multiplexing and quantification of assays are disclosed. Such methods and algorithms can be used in conjunction with any technique which can measure total or targeted amplicon

concentration during amplification, including but not limited to real-time quantitative polymerase chain reaction (qPCR) with fluorescence detection, electrochemical detection or any other real-time detection technique which can quantify the amount of DNA, whether total or specific.

[008] For Point of Care (POC) applications, a single color multiplexing instrument (e.g. detecting single emission wavelength, single channel detection) has the benefits of being simpler, more compact and cheaper to manufacture as compared to a multi-color instrument. Such single color multiplexing instrument can also be faster since it can be more efficient in collecting emitted light and hence reducing the time needed to observe a corresponding signal. The skilled person will know that current multi-color instruments have the extra complexity of using dedicated detection channels per color, where each detection channel can contain a separate excitation light source, reflector, excitation filter, dedicated optical elements (e.g. prisms, gratings, dispersive elements, etc.) and narrow band (e.g. band pass) emission filter, to detect a different emitted spectrum while rejecting other spectra used in the instrument. Due to overlapping emission spectra of dyes used in a multiplex real time qPCR, band pass filters are typically used to differentiate between the various emitted spectral bands, but a portion of the emission from the dye is not used to reduce spectral overlap, thus a less efficient detection can be obtained which can require a longer integration time.

[009] According to some embodiments of the present disclosure, a single color (e.g. single detection channel) multiplexing instrument can be made by using absorbance, interference or combination of filters at the emission side with a wider bandwidth, such as a greater portion of an emission spectrum (e.g. wider range of wavelengths) can be used to detect the amplification process, which can in turn increase a detected signal intensity and thus reduce integration time while conducting fluorescence detection. According to further embodiments of the present disclosure, such single color multiplexing instrument can be used to detect, quantify and verify amplicons emitting at different wavelengths peaks associated to the different dyes and/or probes/primers used for each amplicon. In some cases, only portion of the emitted spectra associated to an amplicon is detected which can be taken into consideration during the quantification phase of the amplification process.

[0010] Testing for a disease is very useful in various applications. However, to ensure quality of a reaction, a positive and negative control is useful to determine whether contamination has occurred and whether the amplification is occurring properly. By conducting qPCR in one well (e.g. cartridge, reaction vessel), one can ease the design of fluidics, save reagents cost, as well as reduce error and contamination. Reducing of errors and contamination can in turn eliminate the requirement for addition of positive and negative controls in separate wells. In case of lyophilized reagents only the eluted DNA has to be added and the reaction can be run in one chamber.

[0011] Embodiments according to the present disclosure can be used in conjunction with various quantitative methods for detecting amplicon concentration using, for example, intercalating dyes which bind to any double string DNA in the assay, or methods using fluorophores attached to specific primers (e.g. Promega Plexor primers) which bind only to a targeted DNA string. Various other detection methods, using for example hybridization-probes such as hydrolysis, molecular beacons, fluorescence resonance energy transfer (FRET), hybridization, etc..., as well as electrochemical methods and electrophoresis methods are compatible with the various embodiments according to the present disclosure.

[0012] The various embodiments according to the present disclosure allow for multiplex detection and quantification of more targets (e.g. different targets) in the following cases (e.g. allows greater multiplexing)

- a. Single fluorophore (e.g. single emission wavelength) for different targets using a single excitation (e.g. single wavelength). In such embodiments a same fluorophore, and thus a single detector, can be used with different sequence specific primers and yet detect different amplicons.
- b. Different fluorophores (e.g. different emission wavelengths) for different targets using a single excitation (e.g. FRET probes which can be all excited using single excitation wavelength).
- c. Different fluorophores for different targets using multiple excitation wavelengths with a single detector and a single emission filter. The single emission filter can be a

wider bandwidth filter to allow the inclusion of larger portions of the emission signal from the various fluorophores (e.g. dyes) such as to obtain an equivalent sum of the emitted intensities.

- d. Different fluorophores for different targets using multiple excitation wavelengths with multiple detectors and emission filters.

[0013] The various embodiments according to the present disclosure further allow for use of DNA binding dyes (e.g. fluorescent) like SYBR Green and FAM, or probes which bind to specific targets, primer attached fluorophores or other DNA binding probes. In further embodiments according to the present disclosure, multiplexing and quantification based on analysis of fluorescence versus temperature curves at various points during an amplification process (e.g. qPCR) other than just at the end of the annealing or extension phases are also provided.

[0014] The various embodiments according to the present disclosure further describe methods for obtaining multiple (e.g. different) quantifications (e.g. of amplicons) by using, for example, a single emission wavelength. It should be noted that the amplicons are obtained from a same assay containing different targets (e.g. DNA sequences or RNA sequences). It should also be noted that for the sake of clarity, the exemplary cases of two different amplicons obtained by amplification of two different targets is presented. Such exemplary cases of two amplicons should not be considered as a limitation of the presented embodiments but rather an exemplary case of the inventive concept as disclosed herein.

[0015] Amplification techniques of target genes or sequences are used to determine (e.g. quantify) an initial concentration of the target genes or sequences. As known by the skilled person, such initial concentration is referred to as, or identified by, the threshold cycle (C_T) which is defined by a detectable initial knee in the amplification curve corresponding to a detectable concentration of amplicons at an amplification cycle (e.g. a qPCR temperature cycle). A detected C_T value can then be correlated to known prior concentrations to derive a concentration of the target genes thus completing the quantification process. Typically derivation of the C_T value is performed after determining a successful (e.g. proper) amplification reaction

which can be verified (e.g. verification process) by conducting, for example, an end point melting curve analysis on the amplicons or using positive and negative controls. In the exemplary case where two different amplicons are amplified (e.g. two different target genes), the end point melting curve analysis can determine whether a single specific amplicon is amplified, whether both amplicons are amplified or whether neither of the amplicons is amplified. In a successful amplification, also referred to as a proper reaction, both amplicons are amplified if the targets for both are present, and therefore one can proceed to find the C_T value for each of the amplicons. In some cases one of the amplification reactions (e.g. creating amplicons) is a control reaction for which the C_T value (e.g. concentration) is known. In other cases concentration of both targets are unknown. According to the various embodiments of the present disclosure, the unknown initial concentrations (e.g. C_T values) can be found in both cases by analyzing the amplitude curve of the multiplexed reaction, also referred to as the sum amplitude curve, which relates to the total number of amplicons per detection cycle. In some cases, the analysis of the sum amplitude curve uses information obtained by analyzing of the melt curve (e.g. melting curve analysis). According to several embodiments of the present application, detailed analysis of the sum amplitude curve and the melt curve can be performed using powerful known mathematical functions and models which the person skilled in the art of signal processing, estimation theory and the like is well aware of.

[0016] When using intercalating dyes, the fluorescence intensity is about proportional to the length of the amplicon (e.g. the longer the amplicon the stronger the fluorescence intensity). According to some embodiments of the present disclosure, methods of analyzing the sum amplitude curve (e.g. corresponding to a total detected intensity) of the multiplexed amplicons is provided which enable to derive the C_T and a corresponding amplitude curve for each of the multiplexed reactions. Such methods are also compatible for the case where primers with attached fluorophores are used. In other embodiment according to the present disclosure more dye molecules can be attached to one primer set vs. the other such as to obtain a higher fluorescence (e.g. amplitude) for one amplicons versus the other as a distinguishing feature of an associated amplification curve. As previously noted, methods according to the various embodiments of the present disclosure can be extended to multiple (e.g. more than two) amplicons. In some embodiments according to the present disclosure, the efficiency of the

reactions can be different or designed to be different such as to limit a corresponding slope and shape of the amplification curve (e.g. of a given reaction) in order to facilitate detection of the various individual curves and corresponding C_T values. In an exemplary embodiment, efficiency in amplification can be changed by using different primer concentrations for the different targets used in the multiplexed reaction such as to obtain individual target amplification curves with different slopes and shapes. Algorithms according to the various embodiments of the present disclosure can be used to separate the different amplification curves, which are superimposed onto each other (e.g. as per a single detection channel). According to further embodiments of the present disclosure, methods to obtain the total fluorescence of each reaction using the melt curve are provided.

[0017] FIGs. 1A-1D show the various possible relationships between the amplitude curves for the case of a multiplexed reaction using two target genes, each identified by a corresponding amplicon (amplicon1, amplicon2) respectively. It is assumed that the reaction is proper, such as the correct genes are amplified at an expected efficiency (e.g. amplitude slope), and verified, for example, via an end point melting curve analysis. For each of the four different cases represented by FIGs. 1A-1D, the total detected signal (e.g. total emitted fluorescence intensity detected via a single detection channel) is represented by the sum amplitude curve (sum), which corresponds to the sum of the individual amplitude curves (amplicon1) and (amplicon2). Furthermore, the derivative of the sum amplitude curve (sum), as well as its double derivative and its normalized double derivative are displayed for each of the four cases.

[0018] In the cases represented by FIGs. 1C and 1D, the amplifications of the two targets occur at differing cycle numbers making it relatively easy to determine the C_T value for each of the reactions by using simple mathematical analysis, such as for example by using the double derivative of the sum amplitude curve, since there is very little overlap (e.g. of the amplification slope) of the two amplification curves as further displayed by the two distinct peaks in the derivative curves. In the cases represented by FIGs. 1C and 1D the slopes associated to the two amplicons are far enough apart such as to allow finding the C_T value for each of the reactions by analyzing the flat portions and/or the portions with slope of the (sum) curve. For example, looking at the graph represented by FIG. 1D, at around cycle 8 a steep slope starts, which can

indicate the start of a detectable amplification, and again at around cycle 19 a second steep slope starts, which can indicate the start of another amplification. By further examining, for example, the initial amplitude of each slope, one can determine that a single amplicon is being amplified during each slope (e.g. by verifying a slope amplitude representing an exponential amplification rate). Melting curve analysis can verify that both target amplicons are amplified. Although C_T points are established, one still needs to associate these points to a specific amplicon of the multiplexed reaction. According to various embodiments of the present disclosure, a combination of sum curve analysis and melting curve analysis can provide said association.

[0019] In the case represented by FIG. 1A, the C_T values are close, and therefore there are no additional peaks in the derivative and double derivative of the (sum) curve as compared to a single amplification curve of a single amplicon, thus rendering determination of the C_T values difficult.

[0020] In the case represented by FIG. 1B, the C_T values are close and there is a single peak in the derivative of the sum curve. However, a closer attention to the derivative curve shows an asymmetrical (bell) curve shape due to a skew of the two C_T points. According to some embodiments of the present disclosure, the amount of asymmetry in the derivative curve can be used to determine the relative position of the two C_T points. Also in the case represented by FIG. 1B, the double derivative has more peaks than the amplification curve of a single amplicon which further indicates a skew of the two C_T points.

[0021] According to the various embodiments of the present disclosure, methods are provided such as to enable derivation of the C_T values and association to specific amplicons of a single detection channel multiplexed reaction for all of the possible cases (e.g. as represented by FIGs. 1A-1D), by mathematically analyzing the sum amplitude curve and/or the melt curve.

[0022] According to an embodiment of the present disclosure, FIG. 2 shows a flowchart of an algorithm for a single color detection of one target with positive and negative controls. Such as in a case of a qPCR reaction, it is here assumed that the reaction is run in a sequence of repeated cycles, wherein further amplification may be obtained with each run of the cycle. The proposed

algorithm provides a method to derive the concentration of the multiplexed target and positive control via detection of the corresponding C_T values irrelevant of their relative distance. The algorithm of FIG. 2 performs the following steps:

1. *At the end of the reaction (e.g. all the cycles) run a melting curve analysis which yields the following:*
 - a. *Which amplicons did amplify, as each amplicons has a different melting temperature (T_M) per design.*
 - b. *What was the amplitude of the fluorescence for each amplified amplicon assuming it reached a plateau level. It also gives the amplitude, which is at the end of the cycles, even if it did not plateau.*
 - c. *The background fluorescence, for example by observing the detected level of fluorescence at the end of the melting curve analysis.*
 - d. *Quality of amplification to some extent, for example by contrasting the observed shape of the melt curve and an expected shape based on known parameters.*
2. *If negative control did amplify then reaction has failed.*
3. *If positive control did not amplify then the reaction has failed.*
4. *If target did not amplify then target is absent/not detectable. Report this and go to End.*
5. *Otherwise do analysis of the sum amplification curve:*
 - a. *Analyze the sum amplitude curve to look for peaks:*
 - i. *Using derivatives and double derivatives of the sum amplitude curve.*
 - ii. *Using various peak detection algorithms known in the art, such as for example CFAR type detection algorithms (e.g. as used in radar theory).*
 - b. *If enough peaks are detected with confidence, then the C_T values are sufficiently distant:*
 - i. *Derive the C_T values of both the amplicon and the positive control. To be noted that the C_T value of the positive control may be known in advance. The C_T value of positive control can be made to be at later cycles, for example, it can be put after the maximum C_T which can be accepted for clinical relevance or acceptance of the reaction. This simplifies finding the C_T value of a valid amplification as the positive control will not affect the determination of the C_T*

value of the target. The amplitude of positive control at or after the plateau phase can also be limited by limiting, for example, the amount of primers.

- ii. Optionally verify the derived C_T values using the distance-based method as described below. (step c.vi.)*
- c. If not enough peaks are detected, then the C_T values are near (assuming amplifications with efficiency as expected/specified):*
 - iii. Optionally normalize amplitudes of target and positive control using the amplitudes obtained from the melting curve analysis.*

(Amplitude may be normalized such as to assume same amplitude for each of the individual amplitude curves as per reference data used to correlate C_T points to known concentrations, otherwise accuracy can be degraded as shown in FIG. 10). Use the total fluorescence intensity (e.g. amplitude) and the points where the melting occurs for each amplicon to estimate the maximum of each individual fluorescence curve.
 - iv. Do a skew analysis on peaks to estimate a C_T difference of the two amplicons. Amplitude information of both amplicons (e.g. target and positive control) as obtained via the melting curve analysis can be used for this step.*
 - v. Assume different values of C_T for target amplification and for positive control amplification and based on the assumed different values generate the corresponding sum amplitude curves. Running various experiments and storing the corresponding data or using mathematical models or a combination of both can obtain these C_T values. If the C_T value of the positive control is known, then it can be fixed and the sum amplitude curves can only be different by the assumed C_T values of the target amplification.*
 - vi. Take the difference of absolute values between the sum amplitude curves (e.g. absolute or squared) based on the assumed C_T values and the actual measured sum amplitude curve. Analyze the result and select the C_T value (or values in the case where positive control value is not known), which is estimated to be the closest to the actual value. Optionally the C_T value corresponding to a minimum distance can be selected as the actual value.*

Optionally the shape of the distance curve/surface/function may be taken into account.

6. *End.*

[0023] Using the algorithm represented by FIG. 2 to implement a single color detection method to amplify a target with both positive and negative controls is attractive as it allows a reliable detection in POC instruments and in commercial machines.

[0024] In multicolor machines (e.g. having multiple detectors), this multiplexing technique (e.g. FIG. 2) can be used for each color thus enabling the following:

- Have positive and negative control on one color and use other colors separately for detection of other targets
- Have positive and negative control for each color and target
- Increase the number of amplicons, which can be detected by two or three fold. (later we show how to increase this even further)

[0025] According to an embodiment of the present disclosure a method for finding amplitude of amplification curves via sum melt curve analysis and finding constituent melt curves from the observed sum melt curve via a single channel detection is provided. FIG. 3 shows an exemplary sum melt curve (MC1+MC2) corresponding to two amplicons (a1, a2) generated through an amplification process. Based on this sum melt curve it is desired to extract its constituent curves (MC1) and (MC2) which are further shown in FIG. 3. The sum melt curve changes slope appreciably near (TM1) and (TM2) which are the melting temperatures of amplicons (a1) and (a2) respectively. According to the observed sum melt curve (MC1+MC2), the slopes at various regions of the curve are known and can be associated to the slopes of the various regions of the individual curves (MC1) and (MC2) as seen in FIG. 3. By defining:

s1bTm: slope of (MC1) before its melt temperature (TM1)

s1aTm: slope of (MC1) after its melt temperature (TM1)

s2bTm: slope of (MC2) before its melt temperature (TM2)

s_{2aTm} : slope of (MC1) after its melt temperature (TM2)

based on the observed sum melt curve (MC1+MC2) and as depicted in FIG. 3, the following expressions are known:

- (a) $s_{1bTm} + s_{2bTm}$
- (b) $s_{1bTm} + s_{2aTm}$
- (c) s_{1bTm}
- (d) s_{1aTm}

These four independent expressions therefore allow to determination of each of the two slopes associated to the constituent melt curves (MC1) and (MC2).

Assuming the amplitudes of the constituent melt curves (MC1) and (MC2) are (A1) and (A2) respectively, then based on the observed single channel sum melt curve, the following amplitudes are known:

- (e) $A1+A2$
- (f) $M1$
- (g) $M2$

Using well known mathematical techniques, the skilled person will know how to estimate (A1) and (A2) by using the relationship of (M1, M2) with (TM1, TM2).

[0026] Finding amplitudes of the various constituent melt curves (e.g. of the sum melt curve) as per the method described above and depicted in FIG. 3, allows to correlate amplitude curves (e.g. C_T values) to specific amplicons, by using for example the relative amplitudes obtained via the sum melt curve analysis provided above (e.g. FIG. 3) and the amplitude curves obtained from the sum amplitude curve as per any one of the various embodiments of the present disclosure. The melt curves can associate an amplicon to a relative amplitude (e.g. via known melt temperature), which can then be correlated to an amplitude of an amplitude curve. For example, and in relation to FIG. 3, amplitude (A2) which corresponds to amplicon (a2) is relatively larger than amplitude

(A1) of amplicon (a1). Therefore, if analysis of the sum amplitude curve corresponding to generation of amplicons (a1, a2), yields in two separate C_T values, the larger value will correspond to amplicons (a2) and the smaller value to amplicon (a1).

[0027] In some cases the slope of the amplitude curves in the plateau phase (e.g. minimal amplification region subsequent to the exponential phase) may be difficult to determine. Therefore, according to an embodiment of the present disclosure the exponential phase only of the assumed amplitude curves (e.g. FIG. 4 later described) may be used for the analysis (e.g. step 5 of the algorithm). Similarly, a phase of an amplitude curve where no amplification is detectable (e.g. relatively flat region of the curve) according to an assumed and/or the actual amplitude curve, can be excluded from the analysis. Such technique of eliminating regions (e.g. phases) of the curve containing minimal information from the analysis step can give a better and more robust analysis result. It should be noted that the region of interest for the analysis is typically the area of exponential amplification of an amplitude curve which is at the vicinity of a corresponding C_T point. Assuming that the amplification is proper (e.g. maximum efficiency and expected amplicons generated), then it is expected that the amplitude change (e.g. amplification) at the vicinity of a C_T point follows an exponential curve, as at the vicinity of a C_T point amplification occurs at a rate of 2^N where N is a number associated to an amplification cycle. The skilled person will not need further explanation on this and will appreciate the benefits of using/analyzing the relevant regions (e.g. slopes) of the amplitude curves in order to detect the various C_T points.

[0028] FIG. 4 shows exemplary amplification curves of two amplicons with C_T points close to each other. As per prior paragraphs, by observing and analyzing a slope (e.g. represented by an efficiency e of the reaction) in FIG. 4) of the sum amplification curve it is possible to directly determine the C_T point of each amplicon. By tracking the exponential phase only of the sum amplitude curve and assuming that each reaction worked properly (e.g. amplified at the proper rate), each of the constituent amplitude curves can be represented as shown in FIG. 4. When the first amplicon starts to be detected above a corresponding C_T value while the second amplicon is not being detected, the efficiency e is close to 1 (e.g. one amplicon amplified properly) as shown in the first segment of FIG. 4. When the second amplicon crosses its corresponding C_T value,

two proper amplification reactions are detectable with each being in a corresponding exponential phase. Thus in the second segment of FIG. 4 the corresponding efficiency (e.g. slope) value is close to 2, showing that both amplifications are in exponential phase. Finding these two initial segments are enough to find the C_T value of each of the two amplicons, based on the fact that an exponential phase of either amplicon has an efficiency (e.g. slope) contribution to the observed sum amplitude curve of approximately 1. In the case where the two C_T values are the same, then the two segments are merged and a single segment with an efficiency value of approximately 2 is observed. In the third segment the efficiency of amplification of one or both amplicons starts decreasing, thus the total observed efficiency also starts decreasing from the value of approximately 2 as an amplification starts approaching a plateau phase. In the third segment the efficiency value (e.g. slope) changes from a value of less than 2 to 0 in the case where both amplifications enter the plateau phase.

[0029] The technique disclosed above to detect C_T values based on the slope of the sum amplitude curve is easily adaptable to cases where C_T values are far apart by easy extension of this technique. This technique is robust since it seeks to find the exponential phase of amplification which occurs during the parts of reaction (e.g. of one of the constituent amplicons) where efficiency is close to 1 and is thus not dependent on the amplification curve taking other shapes due to, for example, limited reagents availability during the later stages of the reaction.

[0030] According to a further embodiment of the present disclosure, the disclosed algorithm for a single color detection of one target with positive and negative controls can be further extended to include more than two targets while keeping both the positive and the negative controls. As for the single target case, the peaks in derivative and double derivative curves and the amplitude (e.g. as detected via the melting curve analysis) can be used to separate the amplitude curves for each of the multiple amplicons from the sum amplitude curve (e.g. steps 5.a. and 5.b. of single amplicons algorithm).

[0031] For the case of multiple targets with one positive control and one negative control, same algorithm as used for the case of single target can be used, with the exception of steps 5.c.v. and 5.c.vi. which may be replaced by the following steps:

For each amplified amplicon assume some C_T values based on prior analysis results.

For all assumed C_T values

Generate all or part of the sum amplitude curve

Find the distance over all or portion of the generated sum amplitude curve to the measured sum amplitude curve (e.g. may calculate distance over a portion such as the exponential phase of the curves)

End for

Select the most probable C_T values (e.g. which minimize the distance)

[0032] According to some embodiments of the present disclosure, the proposed multiplexing algorithm can be used to increase the multiplexing capability of instruments used for amplification of target genes which have multiple optical detection channels. For example by implementing such algorithm in a 4 channel instrument, the instrument can be upgraded to detect 12 targets with 3 targets multiplexed per color using the disclosed multiplexing algorithm. In the case where 4 targets are multiplexed per color using the disclosed algorithm, then the same 4 channel instrument can be upgraded to detect 16 targets. The skilled person will know that such instruments perform all or part of their data analysis of the observed/detected emitted intensity (e.g. fluorescence) based on algorithms implemented in a combination of software and/or firmware code embedded within a processor-controlled hardware of the instrument, such as, for example, the processor-controlled hardware depicted in FIG. 11. Such algorithms perform upon a digital representation of the observed/detected emitted intensity read into a memory module accessible by (or part of) the processor-controlled hardware. Such digital representation can be obtained, for example, via an analog to digital converter module which converts an analog signal representing the observed/detected emitted signal to an equivalent digital representation. The skilled person will realize the possible upgrade to all or portion of such software and/or firmware to include the proposed single channel multiplexed algorithm. In alternative embodiments according to the present disclosure, data observed/detected by an instrument in its standard configuration may be analyzed off-line using the proposed multiplexing algorithm such as to not disturb an existing instrument. In this case the proposed multiplexing algorithm may be entirely implemented in software and/or firmware running on a computer and/or processor-controlled hardware.

[0033] FIG. 11 is an exemplary embodiment of a processor-controlled hardware (10) (e.g. a computer system) as discussed in the previous paragraphs. This processor-controlled hardware comprises a processor (15), a memory bank (20), a local interface bus (35) and one or more Input/Output devices (40). The processor may execute one or more instructions related to the implementation of the discussed mathematical analyses and related algorithms for quantification and verification of the amplification process and as provided by the Operating System (25) based on some executable program stored in the memory (20). These instructions are carried to the processors (20) via the local interface (35) and as dictated by some data interface protocol specific to the local interface and the processor (15). It should be noted that the local interface (35) is a symbolic representation of several elements such as controllers, buffers (caches), drivers, repeaters and receivers that are generally directed at providing address, control, and/or data connections between multiple elements of a processor based system. In some embodiments the processor (15) may be fitted with some local memory (cache) where it can store some of the instructions to be performed for some added execution speed. Execution of the instructions by the processor may require usage of some input/output device (40), such as inputting data from a file stored on a hard disk (e.g. data representing a sum amplitude and/or a sum melting curves), inputting commands from a keyboard, outputting data to a display, or outputting data to a USB flash drive. In some embodiments, the operating system (25) facilitates these tasks by being the central element to gathering the various data and instructions required for the execution of the program and provide these to the microprocessor. In some embodiments the operating system may not exist, and all the tasks are under direct control of the processor (15), although the basic architecture of the processor-controlled hardware device (10) will remain the same as depicted in FIG. 11. In some embodiments a plurality of processors may be used in a parallel configuration for added execution speed. In such a case, the executable program may be specifically tailored to a parallel execution. Also, in some embodiments the processor (15) may execute part of the quantification and/or verification, and some other part may be implemented using dedicated hardware/firmware placed at an Input/Output location accessible by the target hardware (10) via local interface (35) (e.g. dedicated PCB boards for digitizing a detected amplification signal). The processor-controlled hardware (10) may include a plurality of executable program (30), wherein each may run independently or in combination with one another.

[0034] As known by the skilled person, when intercalating dyes are used for detection of an amplification reaction, the amplitude of the fluorescence (e.g. associated with the intercalating dyes) will change with the amplicon length. In the case where sequence specific probes (e.g. bind only to specific targets) are used to monitor an amplification reaction, intercalating dyes can be added to the reaction (e.g. the assay comprising the various reagents) without affecting the monitoring efficiency of the amplification reaction and provide the advantage of allowing a melting curve analysis. The skilled person will know that some sequence specific probes are not compatible with a melting curve analysis thus addition of an intercalating dye can be beneficial in, for example, performing the disclosed single color multiplexed reaction detection algorithm.

[0035] For a probe-based reaction, the number of fluorophores on the probes can be different for different amplicons. The reporter molecules can be different but emit in the same wavelength or in two wavelengths that are substantially the same, such as a same detector can detect both wavelengths with a sufficiently high efficiency. For example FAM, Eva Green and SYBR Green dyes have similar, or substantially same, emission spectra. Such dyes can be used for target specific probes and still allow multiplexed detection of several targets using the disclosed single color multiplexed reaction detection algorithm. This, for example, can allow probe-based detection in a same detection channel. Thus it is possible to get specificity of probes and quantification of different targets using a single detection channel.

[0036] The single color detection algorithm disclosed is also compatible for a multiplexed reaction where different spectra dyes are used. In this case the relatively wide emission spectrum of the various dyes is used to detect all or a portion of the emission spectrum through, for example, a same absorbance filter. For example Texas Red will have a greater portion of its spectrum going through an absorbance filter with a cut-off wavelength of 520 nm (e.g. passes wavelengths above 520nm) than, for example, SYBR Green.

[0037] In the case where FRET probes are used, which have FAM as acceptor dye (e.g. single excitation wavelength) and different donor dyes (e.g. different emission spectra), the amplitude of the different amplicons generated through a multiplexed reaction can be different while being able to use a single excitation wavelength. Distinction in amplitude of the different amplicons

can facilitate correlation of the amplicon specific amplitudes detected via a melting curve analysis, and the C_T points detected from the sum amplitude curves as per the analysis disclosed in the single color multiplexed reaction detection algorithm of this disclosure.

[0038] In some cases different excitation wavelengths are used with different probes designed to emit at different wavelengths. Using different emission wavelengths will yield different amplicon amplitudes for various probes which can be used to further differentiate the various amplicons based on amplitude. For example Alexa Fluor 610 is minimally excited by wavelength of 475 nm (475/25 blue filter) so a blue LED (or a blue filter) can be used to excite SYBR Green only, which yields in detection of amplicons specific to probes with SYBR Green. On the other hand, a wavelength of 410nm can excite both the SYBR Green and the Alexa Fluor dyes, and thus yielding in detection of amplicons specific to probes with both SYBR Green and Alexa Fluor. As depicted in FIG. 8, which was taken from the webpage <http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-Analysis/Labeling-Chemistry/Fluorescence-SpectraViewer.html>, retrieved on August 2, 2013, a detection filter (e.g. 600/200 Em Filter) with a wide enough bandwidth covering a wavelength ranges of [500nm, 700nm] can be chosen to detect emission spectra (e.g. portion of) corresponding to both the SYBR Green and the Alexa Fluor dyes. Detecting emission of both amplicons with one excitation source and then emission of only one amplicon with a second excitation source can allow determining of the emission of each amplicon. As depicted by FIG. 8, one can multiplex using a single detector but different excitation sources and still capture a large portion of the spectrum of dyes in contrast to using bandpass filters specific to each of the emission wavelengths. One can get the amplitude information by getting melting curves with multiple excitation sources in series to get the amplitudes of both. This means select an excitation source, run a multiplexed reaction and analyze reaction using provided algorithm, then select a different excitation source, run a multiplexed reaction and analyze reaction using same algorithm. Multiplexing of the excitation source thus allows increasing detection capability of an instrument for each detection channel.

[0039] To control the amplitude of an amplicon the primers can be limited. For example the positive control can have limited primers so that it plateaus close to a designed value. As

previously mentioned, controlling the amplitude of an amplicon in a multiplexed reaction can facilitate detection of a corresponding CT value as per the disclosed single color multiplexed reaction detection algorithm.

[0040] The initial period of a reaction, where none of the amplicons in the amplification has entered an exponential phase (e.g. corresponding to amplitude curve region with steepest slope), can be treated as background fluorescence and can be subtracted from the total fluorescence waveform (e.g. sum amplitude curve).

[0041] According to one embodiment of the present disclosure, partial melting curve analysis is performed during each cycle of an amplification reaction, such as for example a multiplexed qPCR reaction. Such cycle by cycle partial melting curve analysis can determine the quantification of various amplicons during the course of the amplification reaction. FIGs. 5A and 5B show a typical cycle (e.g. temperature cycle) of an amplification process, whereby a temperature of an assay containing genes to be amplified is subjected to various levels corresponding to a denaturation phase, an annealing phase and an extension phase of a DNA sequence associated to the gene. As represented in FIG. 5A, the temperature levels for the annealing and extension phases are different whereas in the case of FIG. 5B these levels are equal. The skilled person will need no additional information on the inner working of an amplification process and the relevance of the various temperature levels as related to the three main phases of the amplification process. In a typical amplification process, the transition regions from one temperature level of a cycle corresponding to a phase of the cycle to another temperature level corresponding to a different phase of the cycle is not critical to the amplification process and is typically a function of characteristics associated to the heating/cooling elements and a chamber of an associated amplification process instrument. FIGs. 5A and 5B show melting temperatures (TM1, TM2) associated to two different amplicons being amplified. As known by the skilled person these melting temperatures are known a priori and set per design of the target specific primers used in the reaction. According to various embodiments of the present disclosure, melt analysis of the various amplicons of the amplification process, for example as associated to the melt temperatures (TM1, TM2) can be performed at the vicinity of these temperatures, either during a region of ramping up of the temperature or a region of