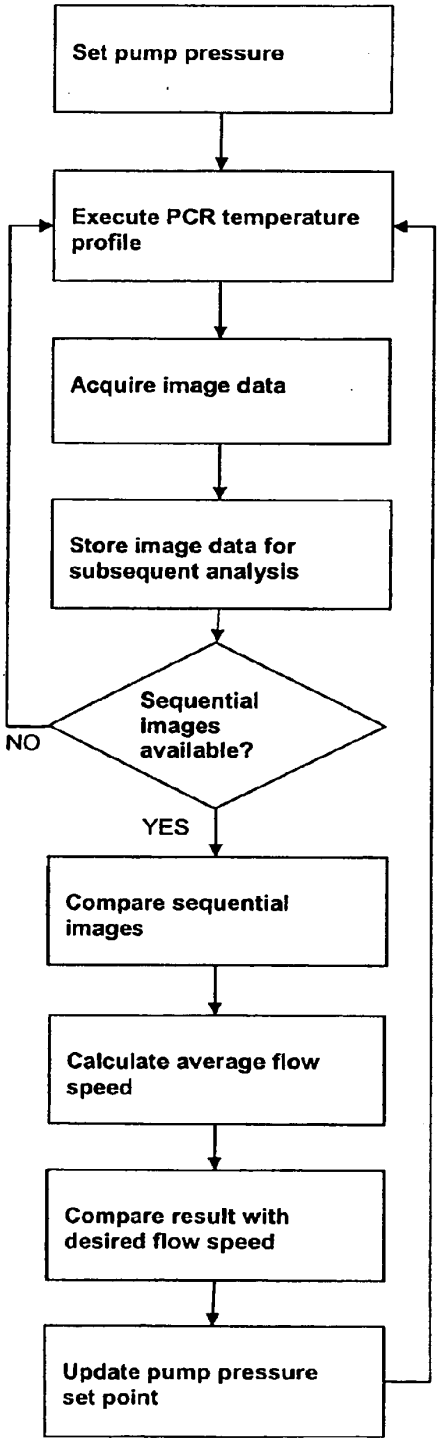

Set pump pressure
Execute PCR temperature profile
Acquire image data
Store image data for subsequent analysis
Sequential images available?
NO
YES
Compare sequential images
Calculate average flow speed
Compare result with desired flow speed
Update pump pressure set point

FIG.7

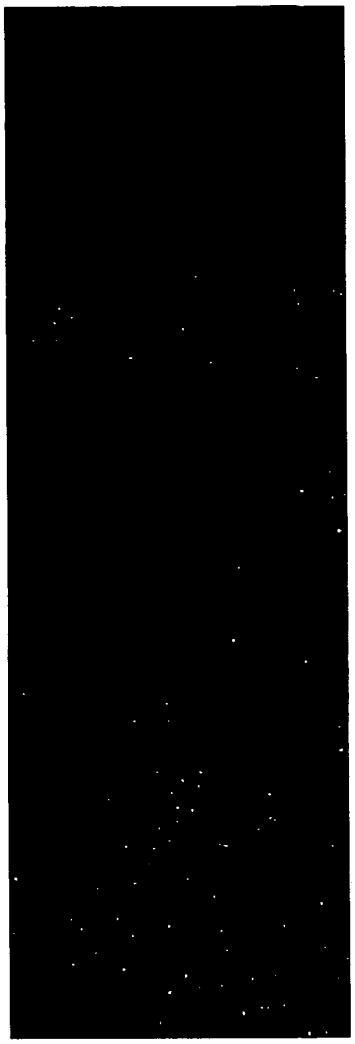


FIG.8A

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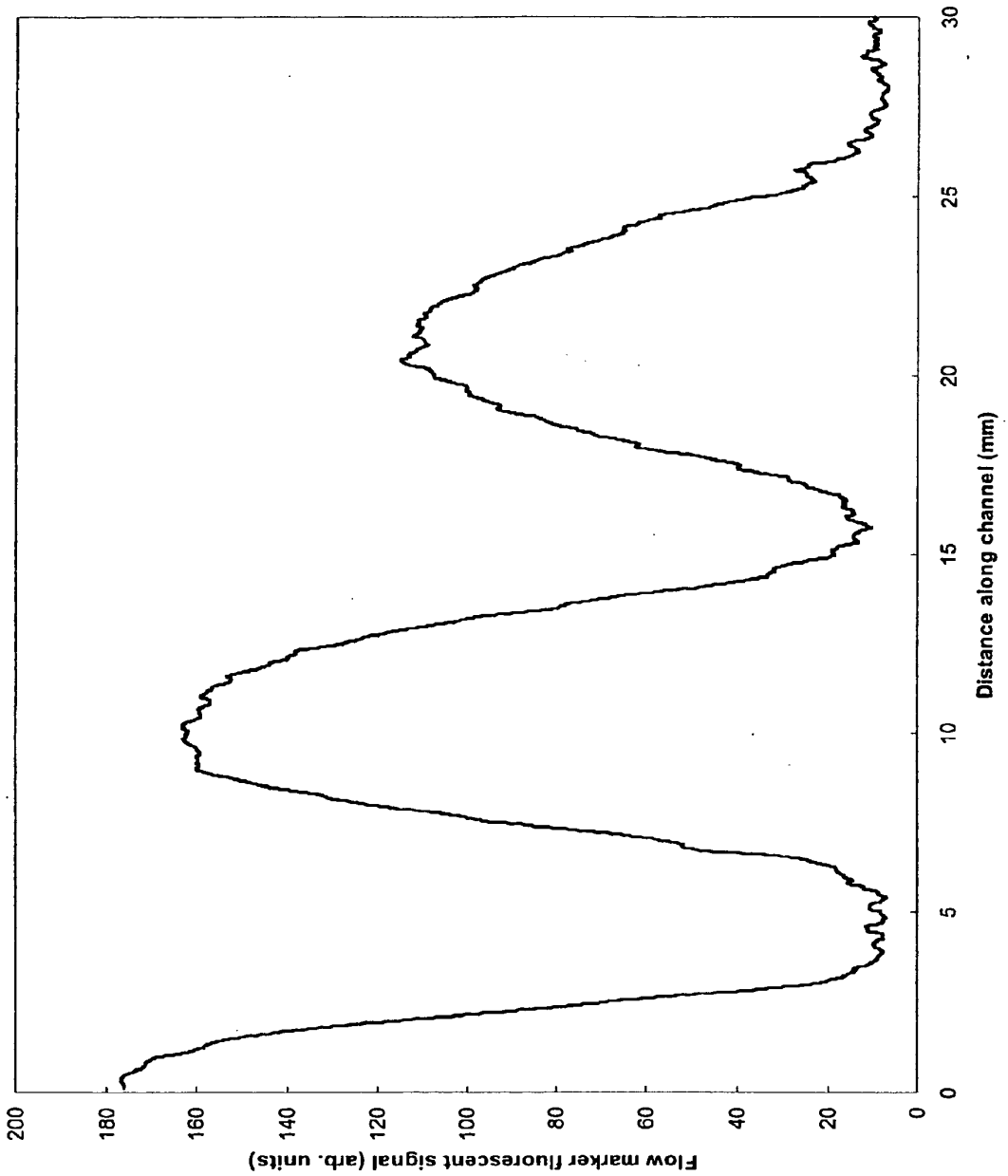

Flow marker fluorescent signal (arb. units)
0
20
40
60
80
100
120
140
160
180
200
0
5
10
15
20
25
30
Distance along channel (mm)

FIG.8B



FIG.9A

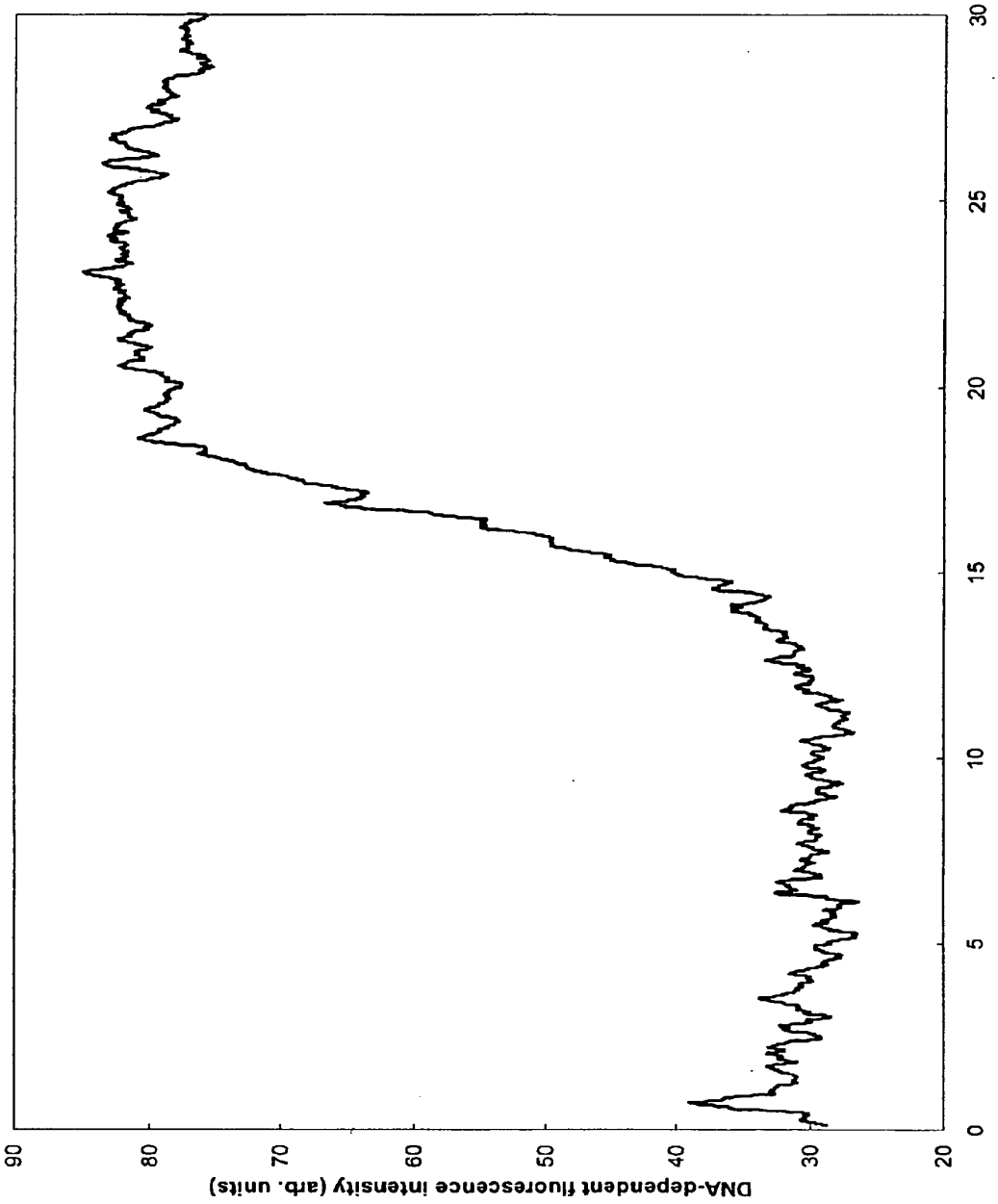

DNA-dependent fluorescence intensity (arb. units)
90
80
70
60
50
40
30
20
0
5
10
15
20
25
30
Distance along channel (mm)

FIG.9B

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常见7种食源性致病菌 xMAP 液相芯片快速筛查方法的建立及应用

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摘要:目的 采用 xMAP 液相悬浮芯片检测方法,建立一管同时检测金黄色葡萄球菌、沙门菌、副溶血弧菌、单增李斯特菌、大肠杆菌 O157:H7、创伤弧菌和空肠弯曲菌的快速筛查方法。方法 根据 GenBank 公布的金黄色葡萄球菌肠毒素 *nuc* 基因、沙门菌侵袭性基因 *ssa*R、副溶血弧菌通用基因 *tox*R、单增李斯特菌的 *hly*A 基因、大肠杆菌 O157:H7的 *rfb*E 基因、创伤弧菌的 *vvh* 基因和空肠弯曲菌的 *gyr*A 基因,设计特异性引物和探针,利用上游引物和生物素标记的下游引物对 7 个靶序列进行不对称实时 PCR 扩增,7 重实时 PCR 扩增产物和制备的 7 种偶联探针的微球在同一体系中进行杂交,最后利用藻红蛋白和生物素的亲和作用报告荧光,从而建立 7 种食源性致病菌的 xMAP 液相悬浮芯片检测法。结果 xMAP 液相悬浮芯片体系检测 140 株菌株,均出现特异性荧光中值信号,7 种食源性致病菌检测互不干扰。DNA 和菌液检出限分别为 1~100ng 和 10^5~10^8cfu/ml 对 56 份各类食品标本进行检测,检出金黄色葡萄球菌 2 份、沙门菌 4 份、副溶血弧菌 7 份、单增李斯特菌 1 份、创伤弧菌 3 份,未检出大肠杆菌 O157:H7和空肠弯曲菌,此结果与传统方法分离结果相一致。结论 xMAP 液相悬浮芯片技术从样品处理到检测结果需 3.5h 左右,该方法可应用于食品安全风险监测中食源性致病菌的快速筛查和食源性致病菌的分型鉴定。

关键词:食源性致病菌 xMAP 液相悬浮芯片技术 多重检测
中图分类号:R155.51 文献标识码:A

Development of a xMAP liquid chip assay for the rapid identification of 7 common foodborne pathogens and its application

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Abstract: Objective To develop an assay for the simultaneous detection of 7 common foodborne pathogens with xMAP liquid chip in a single-tube reaction. **Methods** Seven specific primers and probes were designed and synthesized based on the target gene sequences from GenBank. Target bacterial sequences were amplified by asymmetric PCR. The biotinylated products were hybridized to seven probe beads in a multiplex reaction aud analyzed by using streptavidin conjugated to a fluorescent reporter molecule. The developed liquid chip xMAP assay was used to test 140 strains of bacteria and then 56 food samples. **Results** No cross-reaction aud false signals were observed. The detectiou limit

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was 1 – 100 pg and 10^5 – 10^6 cfu/ml. The results tested by xMAP were in accordance with the traditional culture method. **Conclusion** The processing of xMAP liquid chip assay, including DNA preparation and sample detection, could be finished within 3.5 hours and could be applied to the classification and identification of foodborne pathogens in the food safety monitoring.

Key words: foodborne pathogens, xMAP liquid chip, multiplex detection

食源性疾病已成为当今世界上最关注的公共卫生问题之一[1]。目前食源性致病菌的检测以传统方法为主，操作繁琐、费时耗力[2-3]。虽然借助了一些生化鉴定卡和显色培养基来简化操作流程，但是食品中杂菌多，挑可疑菌落难度大，而且生化反应不稳定，影响结果的准确性。

本文根据食品安全风险监测的食源性致病菌监测内容建立 xMAP 液相悬浮芯片检测 7 种食源性致病菌的检测体系，实现食品中多种食源性致病菌的初步筛查，可提高阳性检出率，同时还可以用于食源性致病菌的鉴定，节省人力和工作时间。

1　材料与方法

1.1　试验用菌株

共 147 株菌株，其中 48 株菌株购自中国药品生物制品检定所（简称检定所），99 株菌株分离自深圳市历年食物中毒或肠道门诊的标本或食品污染物监测的标本（见表 1）。选取 7 株标准菌株或临床分离株用于体系建立和检出限分析，40 株分离株用于特异性试验。56 份食品污染物标本购自于深圳市景田综合市场，用于检测体系的应用和评估。

表 1　实验用菌株

Table 1　The tested strains

细菌类型	细菌数量（株）	来源
沙门氏菌（*Salmonella*）	20	临床分离株
伤寒（*S. typhoid*）、甲型副伤寒（*S. paratyphoid* A）、乙型副伤寒（*S. paratyphoid* B）、丙型副伤寒（*S. paratyphoid* C）、猪霍乱（*S. hogcholera*）、鸭沙门（*S. duck Salmonella*）、马流产（*S. equine abortion*）、阿德莱德（*S. adelaide*）等 34 种血清型沙门菌标准菌株 CMCC50509	各型 1 株，共 34 株	检定所
志贺菌（*Shigella*）	10	临床分离株
痢疾、福氏、鲍氏、宋内氏志贺菌标准菌株（*S. dysentery*、*S. flexneri*、*S. boydii*、*S. sonnei*）	各型 1 株，共 4 株	检定所
产毒性大肠杆菌（*Enterotoxigenic Escherichia coli*）	1	临床分离株
变形杆菌（*Proteusbacillus vulgaris*）	1	临床分离株
单核增生李斯特菌（*Listeria monocytogenes*）	10	临床分离株
单增李斯特标准株（*Listeria monocytogenes* CMCC54001-4）	1	检定所
英诺克李斯特菌（*Listeria innocua*）	3	临床分离株
格氏李斯特菌（*Listeria grayi*）	1	临床分离株
威氏李斯特菌（*Listeria innocua*）	1	临床分离株
副溶血弧菌（*Vibrio Parahaemolyticus*）	20	临床分离株
副溶血弧菌标准株（*Vibrio Parahaemolyticus* CMCC1.165）	1	检定所
创伤弧菌（*Vibrio vulnificus*）	7	食品分离株
霍乱弧菌（*Vibrio cholerae*）	10	临床分离株
乙型溶血性链球菌（*Streptococcus hemolytic-β*）	6	临床分离株
金黄色葡萄球菌（*Staphylococcus areus*）	5	临床分离株
金黄色葡萄球菌标准株（*Staphylococcus areus* CMCC26004-2）	1	检定所
蜡样芽孢杆菌（*Bacillus cereus*）	1	检定所
肉毒梭菌（*Clostridium botalinum*）	1	检定所
产芽孢梭状芽孢杆菌（*Clostridium sporogenes*）	1	检定所
产气肠杆菌（*Enterobacter aerogenes*）	1	检定所
阴沟肠杆菌（*Enterobacter cloacae*）	1	检定所
粘质沙雷氏菌（*Serratia marcescens*）	1	检定所
小肠结肠炎耶尔森菌（*Yersinia enterocolitica*）	1	检定所
空肠弯曲菌（*Campylobacter Jejuni*）	4	食品分离株

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1.2 菌株的分离培养与鉴定

按照中华人民共和国颁布的《食品微生物检验标准》国家检验标准(GB/T4789—2010, GB/T4789—2008)操作。

1.3 试剂与仪器

1.3.1 试剂 细菌基因组纯化试剂盒购自美国QIAGEN生物公司;10×PCR Buffer缓冲液(Mg^{2+} free)、$MgCl_2$、dNTP、rTaq酶购自宝生物工程(大连)有限公司;微球交联试剂盒、链霉素亲和素藻红蛋白(SA-PE)、芯片仪光路校正试剂、芯片仪分析校准试剂等购自美国Bio-Rad公司。

1.3.2 仪器 5417R台式高速冷冻离心机为德国eppendorf公司产品;Thermomixer comfort恒温混匀仪为德国eppendorf公司产品;PTC-200PCR仪为美国Bio-Rad公司产品;菌液比浊仪为法国生物梅里埃公司产品;Milli-Q纯水为美国Millipore公司产品;ND-1000紫外可见分光光度计为美国NanoDrop公司产品;羧基化微球及Luminex200液态芯片仪购于美国Luminex公司。

1.4 引物与探针的设计及合成标记

根据GenBank公布的金黄色葡萄球菌肠毒素*nuc*基因(EF529608)、沙门菌侵袭性基因*ssa*R(CP002614)、副溶血弧菌通用基因*tox*R(AY527396)、单增李斯特菌的*hly*A基因(HM589603)、大肠杆菌O157:H7的*rfb*E基因(JN578668)、创伤弧菌的*vvh*基因(AY046900)和空肠弯曲菌的*gyr*A基因(AY196925),设计7对特异性的引物和7个探针,其中7个下游引物5′端均用Biotin生物素标记,7个探针5′端均用NH_2C_6标记,引物和探针用于Luminex 7种食源性致病菌的检测。沙门菌扩增片段为139bp,金黄色葡萄球菌的扩增片段为124bp,单增李斯特菌扩增片段为112bp,大肠杆菌O157:H7扩增片段为118bp,副溶血弧菌扩增片段为145bp,创伤弧菌扩增片段为101bp,空肠弯曲菌扩增片段为113bp。引物和探针均由宝生物工程(大连)有限公司合成(见表2)。

表2 xMAP液相芯片体系引物和探针设计

Table 2 Primer and probe sequences for xMAP liquid chip

细菌	基因	名称	序列(5′~3′)	扩增长度(bp)
沙门菌	*ssa*R	Sal-F	CTGGCCTGAAGACATAAA	139
(*Salmonella*)		Sal-R	Biotin-AGGTCAATAGCCAGAAAGGCA	
		Sal-probe	NH2C6-TTTTTTTTTTTTTTTGCATTTACGGTGAGTCAGTTA	
单增李斯特菌	*hly*A	lm-F	TGCAAGTCCTAAGACGCCA	112
(*Listeria monocytogenes*)		lm-R	Biotin-CACTGCATCTCCGTGGTATACTAA	
		lm-probe	NH2C6-TTTTTTTTTTTTTTTGGATGAAATCGATAAGTATA TACAA	
金黄色葡萄球菌	*nuc*	Sa-F	CGCAATACGCAAAGAGGTT	124
(*Staphylococcus aureus*)		Sa-R	Biotin-CCACTTCTATTTACGCCGTTATC	
		Sa-probe	NH2C6-TTTTTTTTTTTTTTT GTCTAAGTAGCTCAGCA AATGC	
大肠杆菌O157:H7	*rfb*E	O157-F	ATTGGCATGACGTTATAGGCTAC	118
(*E. coli*O157:*H*7)		O157-R	Biotin-AAATATCAGCAATTTCACGTTTTCG	
		O157-probe	NH2C6-TTTTTTTTTTTTTTTCTATAGGATTAGCCCAGT TAGAA	
副溶血弧菌	*tox*R	vp-F	AAGCGCCAGTAGTACCTGA	145
(*Vibrio parahaemolyticus*)		vp-R	Biotin-CCAATCTGACGGAACTGAGATT	
		vp-probe	NH2C6-TTTTTTTTTTTTTTTGCAAATCGGTAGTAATA CTGCC	
创伤弧菌	*vvh*	vv-F	AGTTAACGGCTGGAGCTGTC	101
(*Vibrio vulnificus*)		vv-R	Biotin-AACGCTCAAACAACGATCGGA	
		vv-probe	NH2C6-TTTTTTTTTTTTTTTCGGCAGTTGGAACCAAGTT	
空肠弯曲菌	*gyr*A	cj-F	AGTGCG(C/A)GCTAAAACTCA	113
(*Campylobacter Jejuni*)		cj-R	Biotin-CAAGCTCTGCAATCTGCTC	
		cj-probe	NH2C6-TTTTTTTTTTTTTTTATCAAACCAATAAAGCT	

1.5 反应模板的制备

空肠弯曲菌于Bolton肉汤在42℃的三气培养,其它6种食源性致病菌分别于LB肉汤中在37℃温箱中隔夜培养。各取增菌液1ml,在

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Eppendorf 高速离心机上 10 000 × *g* 离心 5min，弃上清后加入 200μl 灭菌纯水悬浮，100℃ 煮沸 5min 后于10 000 × *g* 离心 2min，取 5μl 上清作为模板用于 7 重 PCR 实验。

1.6　7 重 xMAP 液相悬浮芯片体系的建立

1.6.1　7 重 PCR 体系建立　通过优化反应条件，建立不对称 7 重实时 PCR 方法，反应总体系为 25μl，内含 5μl 模板，2.5μl 10 × PCR 缓冲液，2mmol/L $MgCl_2$，0.2mmoL/L dNTP，1UTaq 酶，0.04 ~ 0.1μmol/L 引物（上游引物：下游引物 = 1:10）。PCR 反应程序为：95℃ 预变性 10min；94℃变性 30s，55℃退火 45s，72℃延伸 45s，40 个循环；72℃延伸 10min。

1.6.2　微球与探针偶联　取 20μl 打散的各种型号的微球（18#球标记副溶血弧菌、24#球标记空肠弯曲菌、36#球标记沙门氏菌、41#球标记金黄色葡萄球菌、66#球标记创伤弧菌、68#球标记单增李斯特菌、71#球标记大肠杆菌 O157:H7）分别加入到 1.5ml 的聚丙烯微量离心管中，10 000 × *g* 离心 5min 弃去上清，加入 50μl 的核酸偶联缓冲液，全速振荡并用超声波分散约 5min。分别加入 4μl 针对 7 种食源性致病菌的氨基替代的寡核苷酸探针（100pmol/μl）至各种型号微球中。分 3 次各加入 3μl 的多肽耦合剂 EDC 溶液（现用现配），瞬时振荡，定向聚合催化后分别加入 200μl 的核酸偶联洗涤液 1 和 1ml 的核酸偶联洗涤液 2 对各种型号微球进行洗涤，最后加入 100μl 的核酸偶联缓冲液，用血球计时器对微球进行计数。耦连探针后的微球可于 4℃避光保存一年。

1.6.3　7 种食源性致病菌检测　96 孔滤板上加入耦连探针的微球混合液 10μl（200 个球/种），1.5 × 杂交检测缓冲液 33μl 和 7μl PCR 产物，在干浴器中进行 95℃变性 5min，52℃杂交 15min。同时用 1 × 杂交检测缓冲液将链霉素-藻红蛋白（SA-PE）稀释至 4μg/ml，制成新鲜的报告液。向每个反应孔内再加入 25μl 报告混合液，于恒温混匀仪上孵育 5min，杂交温度为 52℃。反应后的 75μl 体系用 Luminex-100 系统读取 MFI 值并进行分析数据。

1.6.4　食源性致病菌 cut off 值的确定　将各样品检测荧光值的均值 MFI 定为检测样品的 cut off 值，作为判断阴阳结果的界值。所检测得到的 MFI ≥ 250 判为阳性，MFI < 100 判为阴性，而 150 ≤ MFI < 250 设置为灰度区间，若检测得到的 MFI 处在这一区间，则样品需要重复试验或采取其它办法进一步验证[4]。

1.7　xMAP 特异性实验

选取 140 株菌株，接种至选择性培养基，培养过夜后提取基因组 DNA 作为 PCR 扩增模板，然后将 PCR 产物和偶联探针的微球进行杂交读取荧光中值，从而验证 xMAP 检测方法的特异性。

1.8　xMAP 7 重检测体系检出限试验

选取伤寒沙门菌标准菌株（CMCC50509）、金黄色葡萄球菌标准菌株（CMCC26004-2）、单增李斯特菌标准菌株（CMCC54001-4）、大肠杆菌 O157:H7临床分离菌株、副溶血弧菌标准菌株（CMCC1.1615）、创伤弧菌临床菌株和空肠弯曲临床菌株作为灵敏度分析的代表菌株，同时做 DNA 检出限的分析及菌液检出限的分析。

DNA 检出限分析：用 QIAamp 公司的 DNA 纯化试剂盒提取上述 7 种食源性致病菌的 DNA 基因组，用 ND-1000 紫外可见光分光光度计测定 DNA 的浓度。将各个模板定量至 100ng/5μl，然后分别采用 10 倍稀释法进行稀释，每个模板做 6 个稀释度，每个稀释度取 5μl 进行 xMAP 检测。

菌液检出限分析：分别对 7 种食源性致病菌进行增菌培养，用比浊仪将各菌液调至 0.5 个麦氏单位（相当于 10^8 数量级），然后进行 10 倍梯度稀释，每个增菌液共做 8 个稀释度，同时相对应的按照中华人民共和国颁布的《食品微生物检验标准》（GB/T4789—2010）做细菌总数计数。最后取比浊后的母液 1ml 在 Eppendorf 离心机上 10 000r/min离心 5min，弃上清后加入 100μl 灭菌纯水悬浮，100℃煮沸 5min 后于 10 000r/min 离心 2min，取上清进行 10 倍梯度稀释，共稀释 5 个梯度。最后取各个梯度的 5μl 模板在 xMAP 仪器上进行菌液灵敏度试验。

1.9　应用

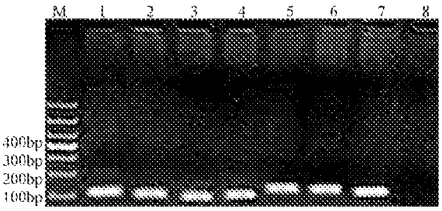
采用传统方法和已建立的 xMAP 液相悬浮芯片方法应用于 56 份各类食品标本的检测，以评估此方法的特异性和灵敏度。

2　结果

2.1　食源性致病菌 7 重 PCR 体系建立

通过调整 Mg^{2+} 含量、rTaq 酶含量、引物含量和退火温度，构建 7 重 PCR 反应体系，电泳结果表明，7 种食源性致病菌株均能扩增出相应大小的特异性目的条带，而对于其他非目的菌株却无相应片段扩出，也未见有非特异性条带，表明所检测的 7 种病原菌之间无交叉反应（见图 1）。

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M:DL1000 DNA Marker;1:大肠杆菌 O157:H7临床菌株(118bp);2:空肠弯曲菌临床菌株(113bp);3:创伤弧菌临床菌株(101bp);4:单增李斯特菌标准菌株(112bp);5:副溶血弧菌标准菌株(145bp);6:沙门菌标准菌株(139bp);7:金黄色葡萄球菌标准菌株(124bp);8:阴性对照

图1 食源性致病菌7重PCR电泳结果

Figure 1 Agarose gel electrophoresis of multiple PCR amplicons of 7 foodborne pathogens

2.2 xMAP液态芯片检测7种食源性致病菌方法的建立

通过调整偶联探针的微球数目、PCR产物含量、PCR反应产物和微球的杂交温度和时间,建立xMAP液相芯片技术对沙门菌、金黄色葡萄球菌、副溶血弧菌、大肠杆菌O157:H7、单增李斯特菌、创伤弧菌、空肠弯曲菌7种食源性致病菌的检测方法,检测结果见表3。

2.3 特异性试验

xMAP液相悬浮芯片技术对7种靶基因的检测结果均为阳性,而非目的菌株检测值均为阴性。与传统检测结果一致,并且7种目的基因之间无交叉反应,表明xMAP液相悬浮芯片方法有较好的特异性。

表3 xMAP液态芯片7菌体系检测结果

Table 3 Results of xMAP liquid chip for the detection of 7 foodborne pathogens

食源性致病菌名称	*Cj*(MFI)	*Lm*(MFI)	*E. coli*O157:*H7*(MFI)	*Sa*(MFI)	*Sal*(MFI)	*Vp*(MFI)	*Vv*(MFI)
NTC	5	8	11	9	13	5	15
Campylobacter Jejuni	395	20	31	15	22.5	22.5	16
Listeria monocytogenes	7	467.5	25.5	11	12	11.5	18
Escherichia coli O157:*H7*	11.5	17	336	9	19.5	11	15
Staphyloccocus aureus	5.5	16	31.5	495	30.5	36	17
Salmonella	5	15	20	15.5	521	13	21
Vibrio parahaemolyticus	8	13	18	7	13	495.5	16.5
Vibrio vulnificus	3	12	89.5	18	17	9	614
cutoff	250	250	250	250	250	250	250

2.4 检出限试验

以不同稀释度的纯DNA和菌液DNA分别为模板进行多重PCR反应,然后扩增产物进行xMAP液相芯片技术检测。在7重检测体系中,沙门氏菌和大肠杆菌O157:H7的灵敏度较高,DNA检出限均为1pg/PCR,创伤弧菌和空肠弯曲菌的DNA检出限均为10pg/PCR,副溶血弧菌、单增李斯特菌、金黄色葡萄球菌的灵敏度较低,DNA检出限为100pg/PCR。对于7菌的菌液检出限,沙门菌和大肠杆菌O157:H7均为10^5cfu/ml,创伤弧菌和空肠弯曲菌均为10^6cfu/ml,副溶血弧菌、单增李斯特菌、金黄色葡萄球菌为10^7cfu/ml。由表4可见,7种食源性致病菌基因组DNA检出限和菌液检出限呈现出很好的一致性。

2.5 应用

利用构建的xMAP液相悬浮芯片体系对56份食品标本进行检测,共检出2份金黄色葡萄球菌、4份沙门菌、7份副溶血弧菌、1份单增李斯特菌、3份创伤弧菌,未检出大肠杆菌O157:H7和空肠弯曲菌,此检测结果和传统方法分离结果相吻合。

表4 xMAP液相芯片检测体系DNA和菌液检出限

Table 4 The limit detection of xMAP assay by DNA and pure culture

食源性致病菌菌名称	DNA检出限(pg)	菌液检出限(cfu/ml)
Salmonella	1	10^5
Escherichia coli O157:*H7*	1	10^5
Vibrio vulnificus	10	10^6
Campylobacter Jejuni	10	10^5
Vibrio parahaemolyticus	100	10^7
Listeria monocytogenes	100	10^7
Staphyloccocus aureus	100	10^7

3 讨论

病原微生物的高通量检测技术是近年发展起来的前沿技术,在病原微生物的检测和预防控制领域具有十分重要的应用价值[5-6]。

液相悬浮芯片技术是基于xMAP(flexible multi-analyte profiling)技术的新型生物芯片技术,有机整合了荧光素编码微球、激光技术、微流体技术、快速信号处理和数据分析系统,不仅可以用于检测机体感染病原体后产生的血清抗体,或直接

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检测病原体抗原，也可以在基因水品上检测病原体的核酸[11]，用途十分广泛[12-13]。针对传统方法操作繁琐，耗时长，灵敏度低等缺陷，本文开展 xMAP 液相悬浮芯片快速检测方法的研究，可用于多个可疑菌落或增菌液的初步鉴定。

相关报道也有对多种病原体的检测[14-15]，胡瑞等[16]建立了 xMAP 液态芯片对 4 种病原微生物的研究，针对 4 种病原微生物设计特异性引物和探针，通过重叠 PCR 扩增目的基因并构建质粒，以重组质粒 DNA 为模板，通过多重 PCR 扩增特异基因，建立 xMAP 液态芯片技术。但没有以菌株 DNA 作为检测对象，也没有应用于实际样品。王子良等[17]针对单增李斯特菌、志贺菌和沙门菌建立液相芯片检测方法，仅检测 3 种致病菌，通量不足，单增李斯特菌、志贺菌和沙门菌三种菌的最低检出限分别是 10^5、10^4 和 10^3 cfu/ml。本文所建立的 xMAP 液相悬浮芯片体系对食品中 7 种食源性致病菌进行同时扩增和检测。结果表明，无交叉荧光信号产生，特异性强。对 7 种食源性致病菌的最低检出限为 1～100pg/PCR 和 10^5～10^7cfu/ml 范围内，可用于食品中食源性致病菌的初步鉴定。但是与王子良等[13]所建立的单一病原检测体系进行比较，由于微球偶联的效率不高，导致检测敏感性整体偏低，还有待进一步优化多重 PCR 反应体系条件和微球偶联效率，以提高灵敏度。

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类型	国别以及代码[11] 给出的文献号	代码[43]或[45] 给出的日期	IPC 分类号	相关的段落 和/或图号	涉及的权 利要求
X	CN101570782A	2009-11-04	C12Q1/68	说明书第 2 页第 4 段至第 4 页第 7 段，附图 3,6-8	1-2
A	CN102150043A	2011-08-10	G01N33/50	全文	1-20
A	US2009297525A1	2009-12-03	A61K31/7088	全文	1-20
A	WO2008005248A2	2008-01-10	G06T7/20	全文	1-20
T	CN104271767A	2015-01-07	C12Q1/68	全文	1-20
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相关非专利文献

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A	卫生研究,第 01 期	2012-01-30	吕东月;石晓路;陈妙龄;吴平芳;贺连华;李迎慧;林一曼;邱亚群;扈庆华;,常见 7 种食源性致病菌 xMAP 液相芯片快速筛查方法的建立及应用	全文	1-20
T	《Analytical Chemistry》,第 85 卷,第 16 期	2013-06-13	Aditya Rajagopal 等,Supercolor Coding Methods for Large-Scale Multiplexing of Biochemical Assays	7629-7636	1-20
类型	网址	网络发布日或公开日	作者姓名和网页标题	相关部分	涉及的权利要求

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2024 年 01 月 05 日

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UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/352,112	**02/01/2024 06:14:07 PM Z ET**	**88DG-373535-US**

Title of Invention

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	3145	FILED BY	Annie Wong
PATENT CENTER #	64180280	FILING DATE	07/13/2023
CUSTOMER #	30764	FIRST NAMED INVENTOR	Emil P. KARTALOV
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	David Heisey

Documents

TOTAL DOCUMENTS: 13

DOCUMENT		PAGES	DESCRIPTION	SIZE (KB)
NFOA_Response_18352112.pdf		14	-	92 KB
NFOA_Response_18352112-A....pdf	(1-1)	1	Amendment/Request for Reconsideration-After Non-Final Rejection	27 KB
NFOA_Response_18352112-CLM.pdf	(2-7)	6	Claims	45 KB
NFOA_Response_18352112-REM.pdf	(8-14)	7	Applicant Arguments/Remarks Made in an Amendment	58 KB
IDS-3_18352112.pdf		4	Information Disclosure Statement (IDS) Form (SB08)	516 KB
CN102150043A_pt2.pdf		47	Foreign Reference	19990 KB

CN102150043A_pt1.pdf	145	Foreign Reference	12700 KB
CN104271767A_pt1.pdf	68	Foreign Reference	8174 KB
CN104271767A_pt2-.pdf	59	Foreign Reference	16917 KB
CN107326085A_pt1.pdf	69	Foreign Reference	5442 KB
CN107326085A_pt2-.pdf	89	Foreign Reference	17961 KB
WO2008-005248A2.pdf	85	Foreign Reference	2997 KB
SR1_CN2022103893474_05 Jan2024.pdf	3	Other reference-Patent/Application/Search Documents	392 KB
LU.pdf	6	Non Patent Literature	2578 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
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NFOA_Response_18352112-REM.pdf	6CAFF2BD476BBA898F430BDCAA86E6010D042E2A7199B5F1353C2889B782D187DF2632AB07726F92FD4EB78A677A51E3D363CF147FD3B6E89751B978D1BB07CB
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CN102150043A_pt2.pdf	83126F6F70FC1B8A95A90B172FBCD2C30748520A6C5DDE84346B2B1013959FAD6632A884DC1C04C94A0CD22FA6BF42FABD81C03D8BAB5EE2FE10E456F0983BA1
CN102150043A_pt1.pdf	4D46EA97727D0985A7DA82F06B888B526F7BD189D26F2FC64BA6BFA8A63463D2A8D218D76C72273E579B6BABBCC0C0704191562FD130878EE845CD7A1EC02606
CN104271767A_pt1.pdf	76A91F189D17B5698478BF7E0F3BEF9369CC948FBFAC6AF2CB97F7C1835B8FBE1A9F63F21A3126605228727E68CD6E2B6E

	702E009459D5DB1026AABB0C886954
CN104271767A_pt2-.pdf	132C53EB361AC740D73DCB1D2DE5E2F97EC5713BD795DDC0885B293574E8F3333278B3A2F3D7037200DE1060938EA92416CA525D334E26D05FA6D952C0998387
CN107326085A_pt1.pdf	E81272D9E1CA8C1BBA6CB73255851A5954444299197857F19DDCE2E9B076D9EB30FDE01D5ED58498E500AA9D1977C6314990F34E1CF8E3EA2C00F6C18DA39006
CN107326085A_pt2-.pdf	13EBF8D39893A458ACA99D46FFDBDBF905D611783C474E823AED702446690575971781A2018DDB2DEAFBAD82E173311E569FEC106DDF444D0F15FA36C56C5CAC
WO2008-005248A2.pdf	230A4342F73E6A0900ABCFE9965375271988679EBB5DA94F4660049B7C8EBDFE3745A1F2D19ADA13A73874701342606D3ADB104E3055414DFC3285EF07BE30F2
SR1_CN2022103893474_05Jan2024.pdf	6E0F87CA7899C3F510B0E73B00DDABDE3E4B70CC48B38787E4530B1590D073E05F3A262D4FEFCF72E6534CD82B9E44D4B463B875C3267D0EAB4671B7BE6F13D7
LU.pdf	FCB2446BF2B9B08B3708160FAEB45589367C11F87B19B11AB9A1318E100A6FC351407E811E21CBD1C50AD86D0B32A7A8F9C7C32A15D39B699D136D802262B024

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New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

PATENT
Docket No.: 88DG-373535-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

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

Commissioner:

RESPONSE TO OFFICE ACTION

In response to the non-final Office Action mailed January 5, 2024, the period for response ending on April 5, 2024, being submitted with **no** extension of time, Applicant respectfully requests the Examiner to reconsider this Application in light of the following.

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

Remarks begin on page 11 of this paper.

AMENDMENTS TO THE CLAIMS:

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

1. (Currently Amended) A system comprising:

a sample chamber configured to house a sample and analyte-specific reagent mixtures of analyte-specific hybridization probes and multiple fluorophores;

a multi-channel detector to detect:

a first electromagnetic signal at a first wavelength from the sample chamber, the first electromagnetic signal generated by excitement of a first fluorophore of the multiple fluorophores;

a second electromagnetic signal at a second wavelength from the sample chamber, the second electromagnetic signal generated by excitement of a second fluorophore of the multiple fluorophores;

a third electromagnetic signal at a third wavelength from the sample chamber, the third electromagnetic signal generated by excitement of a third fluorophore of the multiple fluorophores;

a fourth electromagnetic signal at a fourth wavelength from the sample chamber, the fourth electromagnetic signal generated by excitement of a fourth fluorophore of the multiple fluorophores;

a processor controlled analyzer to receive, from the multi-channel detector, a cumulative signal based on the first, second, third, and fourth electromagnetic signals and apply a decoding matrix to the cumulative signal to unambiguously detect the presence or absence of each of M analytes by associating, for each analyte, a first value in a first component of the cumulative signal and a second value in a second component of the cumulative signal, wherein each first value is an intensity or range of intensities and each second value is a wavelength or a range of wavelengths, and wherein the second values comprise the first, second, third, and fourth wavelengths, and the determination is made without mass spectrometry or melting curve analysis; ~~and~~

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wherein M is greater than the ~~the~~ number of the second values used to encode the analytes (C), the multi-channel detector comprises C channels, and M and C are positive integers.

2. (Previously Presented) The system of claim 1, wherein the multi-channel detector is further configured to detect:

a fifth electromagnetic signal at a fifth wavelength from the sample chamber, the fifth electromagnetic signal generated by excitement of a fifth fluorophore of the multiple fluorophores; and

a sixth electromagnetic signal at a sixth wavelength from the sample chamber, the sixth electromagnetic signal generated by excitement of a sixth fluorophore of the multiple fluorophores.

3. (Currently Amended) The system of claim 1, wherein for the positive integer M,

$M=C*\log2 (F+1)$, ~~wherein~~

F is a positive integer and is equal to the maximum cumulative intensity of the first component of the signal, for any second value, when all of the analytes are present, and

C=4, 5, or 6.

4. (Previously Presented) The system of claim 3, wherein F=3.

5. (Previously Presented) The system of claim 1, wherein the multiple fluorophores is selected from the group consisting of a blue fluorophore, a green fluorophore, a yellow fluorophore, a red fluorophore, and any combinations thereof.

6. (Previously Presented) The system of claim 1, wherein the multiple fluorophores is selected from the group consisting of a fluorescein amidite (FAM), a cyanine 3 (Cy3), a carboxy-X-rhodamine (ROX), a cyanine 5 (Cy5), a cyanine 5.5 (Cy5.5), and any combinations thereof.

7. (Previously Presented) The system of claim 1, wherein the multiple fluorophores has a maximum excitation wavelength selected from the group consisting of about 494 nm, about 550 nm, about 567 nm, about 650 nm, about 675 nm, and any combinations thereof.

8. (Previously Presented) The system of claim 1, wherein the multiple fluorophores has a maximum emission wavelength selected from the group consisting of about 518 nm, about 565 nm, about 591 nm, about 670 nm, about 697 nm, and any combinations thereof.

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9. (Previously Presented) The system of claim 1, wherein the analyte-specific hybridization probes are labeled with a fluorophore of the multiple fluorophores.

10. (Previously Presented) The system of claim 1, wherein the detector comprises four or six band pass filters.

11. (Previously Presented) The system of claim 1, wherein the detector comprises band pass filters that enable the detection of blue, green, and red fluorescent signals; and wherein the detector comprises band pass filters that enable the detection of ranges selected from the group consisting of from about 483 nm to about 533 nm, from about 523 nm to about 568 nm, from about 558 nm to about 610 nm, from about 615 nm to about 670 nm, and any combinations thereof.

12. (Previously Presented) The system of claim 1, wherein the multi-channel detector comprises four or six photodetectors.

13. (Previously Presented) The system of claim 1, wherein the multi-channel detector comprises photodetectors that enable the detection of blue, green, and red fluorescent signals.

14. (Currently Amended) The system of claim 1, wherein the multi-channel detector comprises ~~photodetecors~~photodetectors that enable the detection ranges selected from the group consisting of from about 483 nm to about 533 nm, from about 523 nm to about 568 nm, from about 558 nm to about 610 nm, from about 615 nm to about 670 nm, and any combinations thereof.

15. (Previously Presented) The system of claim 1, wherein the sample comprises a droplet.

16. (Previously Presented) The system of claim 15, wherein the droplet is housed in a dynamic array.

17. (Previously Presented) The system of claim 2, wherein each of the six fluorophores is attached to a hybridization probe.

18. (Previously Presented) The system of claim 1, further comprising a display coupled to the processor controlled analyzer to visualize a plot of the first and second values.

19. (Currently Amended) A system comprising:

a processor;

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a display; and

a non-transitory computer readable medium storing instructions thereon that, when executed by the processor, cause the processor to:

obtain cumulative signal data from a digital PCR instrument with a sample volume, the digital PCR instrument comprising a light source and a multichannel detector with C channels, and the sample volume comprising M fluorescently labeled polynucleotide analytes;

apply a decoding matrix to the cumulative signal data to unambiguously determine the presence or absence of each of the M fluorescently labeled polynucleotide analytes by associating, for each fluorescently labeled polynucleotide analyte, a first value in a first component of the cumulative signal data and a second value in a second component of the cumulative signal data, wherein each first value is an intensity or range of intensities and each second value is a wavelength or a range of wavelengths,

wherein for a positive integer M, $M=C*\log_2 (F+1)$,

F is the maximum cumulative intensity of the first component of the cumulative signal data, for any second value, when all of the analytes are present, and

M, C, and F are each positive integers; and

plot, on the display, a representation of the first value and the second value;

wherein the cumulative signal data comprises:

a first electromagnetic signal at a first wavelength from the sample volume, the first electromagnetic signal generated by excitement of a first fluorescently labeled polynucleotide analyte of the six fluorescently labeled polynucleotide analytes;

a second electromagnetic signal at a second wavelength from the sample volume, the second electromagnetic signal generated by excitement of a second fluorescently labeled polynucleotide analyte of the six fluorescently labeled polynucleotide analytes;

a third electromagnetic signal at a third wavelength from the sample volume, the third electromagnetic signal generated by excitement of a third fluorescently labeled polynucleotide analyte of the six fluorescently labeled polynucleotide analytes;

a fourth electromagnetic signal at a fourth wavelength from the sample volume, the fourth electromagnetic signal generated by excitement of a fourth first fluorescently labeled polynucleotide analyte of the six fluorescently labeled polynucleotide analytes; and

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wherein the second values comprise the first, second, third, and fourth wavelengths, and the determination is made without mass spectrometry or melting curve analysis.

20. (Currently Amended) A reaction mixture for unambiguously detecting a presence or absence of a plurality of non-immobilized polynucleotide analytes of a single sample, the reaction mixture comprising:

a plurality of non-immobilized hybridization probes,

wherein the reaction mixture is subjected to conditions such that the plurality of polynucleotide analytes are amplified;

wherein at least one of the plurality of oligonucleotide primers is used to amplify a region complementary of at least one of the plurality of hybridization probes;

wherein the plurality of hybridization probes comprise a first hybridization probe conjugated to a first fluorophore and a second fluorophore,

wherein the first fluorophore and the second fluorophore, when excited by a light source, emit light of different wavelengths, and

~~optionally~~ the plurality of hybridization probes further comprises a third hybridization probe and a fourth hybridization probe, wherein the third hybridization probe is conjugated to a third fluorophore and the fourth hybridization is conjugated to a fourth fluorophore,

wherein the third fluorophore and the fourth fluorophore, when excited by a light source, emit light of a same wavelength, and

wherein the third hybridization probe and the fourth hybridization probe are present at different concentrations in the reaction mixture;

optionally the plurality of hybridization probes further comprises a fifth hybridization probe and a sixth hybridization probe, wherein the fifth hybridization probe is conjugated to a fifth fluorophore and the sixth hybridization is conjugated to a sixth fluorophore;

wherein the fifth fluorophore and the sixth fluorophore, when excited by a light source, emit light of a same wavelength, and

wherein the fifth hybridization probe and the six hybridization probe are present at different concentrations in the reaction mixture.

21. (Previously Presented) The reaction mixture of claim 20, wherein the reactions mixture is separated into droplets.

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22. (Previously Presented) The reaction mixture of claim 20, wherein the first fluorophore, the second fluorophore, the third fluorophore or the fourth fluorophore is selected from the group consisting of a fluorescein amidite (FAM), a cyanine 3 (CY3), a carboxy-X-rhodamine (ROX), a cyanine 5 (CY5), a cyanine 5.5 (CY5.5), and any combinations thereof.

23. (Currently Amended) The reaction mixture of claim 20, wherein the first fluorophore, the second fluorophore, the third ~~fluororphore~~fluorophore or the fourth fluorophore has a maximum ~~exitation~~excitation wavelength selected from the group consisting of about 494 nm, about 550 nm, about 567 nm, about 650 nm, about 675 nm, and any combinations thereof.

24. (Currently Amended) The reaction mixture of claim 20, wherein the first fluorophore, the second fluorophore, the third ~~fluororphore~~fluorophore or the fourth fluorophore has a maximum emission wavelength selected from the group consisting of about 518 nm, about 565 nm, about 591 nm, about 670 nm, about 697 nm, and any combinations thereof.

25. (Previously Presented) The reaction mixture of claim 20, wherein a ratio of concentration between the first hybridization probe and the second hybridization probe is between 0.3 and 0.7 mol/mol.

26. (Previously Presented) The reaction mixture of claim 20, wherein the reaction mixture further comprises a polymerase having 5′ to 3′ exonuclease activity and primers specific for the plurality of polynucleotide analytes.

27. (Previously Presented) The reaction mixture of claim 24, wherein the first fluorophore, the second fluorophore, the third fluorophore, the fourth fluorophore, the fifth fluorophore, or the sixth fluorophore comprise multiple fluorophores.

28. (Previously Presented) The reaction mixture of claim 20, wherein one of the plurality of hybridization probes further comprise a fluorescent quencher.

29. (Previously Presented) The reaction mixture of claim 20, wherein the first hybridization probe and the second hybridization probe are present at different concentrations in the reaction mixture.

30. (Previously Presented) A kit for the unambiguously detecting a presence or absence of a plurality of polynucleotide analytes of a single sample comprising the components claim 20 and instructions for the use of the kit.

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REMARKS

In the non-final Office Action, the Examiner:

I. rejects claims 3 and 19 under 35 U.S.C. § 112(b) as allegedly being indefinite, *Office Action* at 2;

II. rejects claims 3 and 19 under 35 U.S.C. § 112(a) as allegedly failing to comply with the written description requirement, *Office Action* at 3;

III. rejects claims 1-2, 5-14, 17-18, 20, 22-24, 27, and 29-30 under 35 U.S.C. § 103 as being rendered obvious by Han et al., Nature Biotechnology, volume 19, 2001, pages 631-635 (*Han*) and Published Application No. WO 2004/087950 A2 (*Sagner*), *Office Action* at 5;

IV. rejects claims 15-16, 21, 25-26, and 28 under 35 U.S.C. § 103 as being rendered obvious by *Han*, *Sagner*, and Vogelstein et al., PNAS, volume 96, 1999, pages 9236-9241 (*Vogelstein*), *Office Action* at 8-9;

V. rejects claim 19 for nonstatutory double patenting over claim 1 of U.S. Patent No. 8,838,394 B2 in view of *Sagner*;

VI. rejects claims 1 and 20 for nonstatutory double patenting over claim 1 of U.S. Patent No. 10,068,051 B2 in view of *Sagner*;

VII. rejects claims 1, 15, 20, and 21 for nonstatutory double patenting over claim 1 of U.S. Patent No. 10,770,170 B2 in view of *Sagner*; and

VIII. rejects claims 1 and 20 for nonstatutory double patenting over claim 22 of copending U.S. Application No. 16/937,464 in view of *Sagner*.

To expedite allowance of this Application, Applicant has made clarifying amendments to claims 1, 3, and 19. Applicant has also amended Claim 20 to delete the word "optionally." No new matter has been added. Applicant respectfully requests reconsideration of this application, and the timely allowance of the pending claims for at least the following reasons.

I. The Rejection of Claims 3 and 19 under 35 U.S.C. § 112(b)

The Examiner rejects claims 3 and 19 under 35 U.S.C. § 112(b) as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor regards as the invention. In particular, the Examiner alleges that "[i]t is unclear as to the meets and bounds of M being an integer when F+1 [in the equation $M=C*\log_2(F+1)$] is not a power of 2" (Office Action at page 3). Applicant respectfully disagrees.

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As the Examiner correctly recognizes, "M," the number of analytes, is a positive integer. See Claim 1 "wherein … M and C are positive integers." Thus, a person of ordinary skill in the art would have understood that the "F" in Claim 3, which depends on Claim 1, would be the positive integer values such that M in the equation $M=C*\log_2(F+1)$ would be a positive integer. This is consistent with Examiner's correct determination that Claim 4 (referred to as Claim 20 in the Office Action at pages 3 and 4) fully complies with 35 U.S.C. § 112(b) and is allowable (see also Office Action at page 15). Nevertheless, in an effort to accelerate prosecution of this Application, Applicant has amended the original language of claim 3 to:

"wherein for the positive integer M,

$M=C*\log_2(F+1)$,

~~wherein~~ F is a positive integer and is equal to the maximum cumulative intensity of the first component of the signal, for any second value, when all of the analytes are present, and

C=4, 5, or 6."

Thus, it would be clear a person of ordinary skill in the art that in the equation $M=C*\log_2(F+1)$, M is a positive integer and that the meets and bounds of M is clear. For at least these reason, reconsideration and withdrawal of the rejection are respectfully requested.

Similarly, Claim 19 is not indefinite for the same reasons that Claim 3 is not indefinite. Additionally, in an effort to accelerate prosecution of this Application, Applicant has made similar amendments to Claim 19 to include the language "for a positive integer M." Accordingly, it would be clear a person of ordinary skill in the art that in the equation $M=C*\log_2(F+1)$, M is a positive integer and that the meets and bounds of M is clear. For at least these reason, reconsideration and withdrawal of the rejection are respectfully requested.

II. The Rejection of Claims 3 and 19 under 35 U.S.C. § 112(a)

The Examiner rejects claims 3 and 19 under 35 U.S.C. § 112(a) as allegedly to not enabling any person skilled in the art to make and use the invention. In particular, the Examiner alleges that "[i]t is unclear as to the meets and bounds of M being an integer when F+1 [in the equation $M=C*\log_2(F+1)$] is not a power of 2" (Office Action at page 4). Applicant respectfully disagrees for the same reasons as Section I. For at least the reasons and amendments in Section I, reconsideration and withdrawal of the rejection are respectfully requested.

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III. Rejection of claims 1-2, 5-14, 17-18, 20, 22-24, 27, and 29-30 under 35 U.S.C. § 103

The Examiner rejects claims 1-2, 5-14, 17-18, 20, 22-24, 27, and 29-30 under 35 U.S.C. § 103 as being rendered obvious by *Han* and *Sagner*. Applicant respectfully disagrees.

A. Claims 1-2, 5-14, and 17-18

Independent Claim 1 is directed to "a system comprising…a processor controlled analyzer to receive, from the multi-channel detector, **a cumulative signal** based on the first, second, third, and fourth electromagnetic signals and apply a decoding matrix to **the cumulative signal** to unambiguously detect the presence or absence of each of M analytes by associating, for each analyte, a first value in a first component of **the cumulative signal** and a second value in a second component of **the cumulative signal**, wherein each first value is an intensity or range of intensities and each second value is a wavelength or a range of wavelengths, and wherein the second values comprise the first, second, third, and fourth wavelengths, and the determination is made without mass spectrometry or melting curve analysis…"

In stark contrast, *Han* teaches a method of multiplexing in which "[o]ptical readout is accomplished by measuring the fluorescence spectra of ***single beads***." (Han at Figure 1, page 632, emphasis added). Furthermore, Han teaches the ***immobilization of single-color signal beads*** on a polylysine-coated glass slide as expressly illustrated in Figure 2 (Han, at Figure 2, Page 632). Han's **single-bead** multiplex approach is again emphasized in Fig. 5, where Han describes the necessary coding scheme to identify the presence and abundance **of each individual bead** (i.e., single targets). Specifically, Fig. 5 on page 634 teaches: "To demonstrate the use of QD-tagged beads for biological assays, we designed a model DNA hybridization system using oligonucleotide probes and triple-color encoded beads, as shown in Figure 5. **Target DNA molecules** are either directly labeled with a fluorescent dye or with a biotin (for binding to fluorescently tagged avidin). **Optical spectroscopy at the single-bead level** yields **both the coding and the target signals**. The coding signals identify **the DNA sequence**, whereas the target signal **indicates the presence and the abundance** of **that sequence** (emphasis added)."

Therefore, *Han* does not teach or suggest a system, as claimed, in which a processor controlled analyzer that receives **a cumulative signal** and apply a decoding matrix to the cumulative signal to unambiguously detect the presence or absence of each M by associating, for

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each analyte, a first value in a first component of the cumulative signal and a second value in a second component of the cumulative signal.

The Office Action failed to explain how *Sagner* could overcome the deficiencies of *Han*. *Sagner* discloses "a system for performing multi-color real time PCT, comprising a flexible real time PCT instrument and a specific composition or reaction mixture for performing multiplex PCR [including] at least 3, preferably 4-5 and most preferably exactly 4 pairs of FRET hybridization probes" (*Sagner* at Abstract). Cited sections of *Sagner* does not explain how a person of ordinary skill in the art, in view of *Han* and *Sagner*, would arrive at the claimed embodiments of Claim 1. Section 2142 of the MPEP citing *Ex parte Clapp*, 227 USPQ 972, 973 (Bd. Pat. App. & Inter. 1985) states: "[t]o support the conclusion that the claimed invention is directed to obvious subject matter, either the references must expressly or impliedly suggest the claimed invention or the examiner must present a convincing line of reasoning as to why the artisan would have found the claimed invention to have been obvious in light of the teachings of the references." Section 2143 of the MPEP, citing *In re Wilson*, 424 F.2d 1382, 1385, 165 USPQ 494, 496 (CCPA 1970), further instructs that "[a]ll words in a claim must be considered in judging the patentability of that claim against the prior art." For at least these reasons, Applicant respectfully request the Examiner to explain how the system claim comprising a processor controlled analyzer that receives **a cumulative signal** and apply a decoding matrix to the cumulative signal to unambiguously detect the presence or absence of each M by associating, for each analyte, a first value in a first component of the cumulative signal and a second value in a second component of the cumulative signal would be obvious in view of the combination of *Han* and *Sagner*. In the absence of such an explanation supporting a *prima facie* obviousness rejection, Applicant respectfully requests withdrawal of the rejection with respect to independent system Claim 1, and its dependent claims 2, 5-14, and 17-18.

B. Claims 20, 22-24, 27, and 29-30

Independent Claim 20 is directed to "[a] reaction mixture for unambiguously detecting a presence or absence of a plurality of **non-immobilized polynucleotide analytes of a single sample**, the reaction mixture comprising: a plurality of **non-immobilized hybridization probes**." In stark contrast, as explained above, *Han* teaches a method of multiplexing involving the ***immobilization of single-color signal beads*** on a polylysine-coated glass slide as expressly

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illustrated in Figure 2 (Han, at Figure 2, Page 632). Therefore, *Han* does not teach or suggest a reaction mixture for unambiguously detecting a plurality of **non-immobilized** polynucleotide analytes of a single sample. The Office Action also failed to explain how *Sagner* could overcome the deficiencies of *Han*. Thus, in the absence of an explanation supporting a *prima facie* obviousness rejection, Applicant respectfully requests withdrawal of the rejection with respect to independent reaction mixture Claim 20, and its dependent claims 2, 22-24, 27, and 29-30.

IV. Rejection of claims 15-16, 21, 25-26, and 28 under 35 U.S.C. § 103

The Examiner rejects claims 15-16, 21, 25-26, and 28 under 35 U.S.C. § 103 as being rendered obvious by *Han*, *Sagner*, and *Vogelstein*. Applicant respectfully disagrees.

Claims 15-16 depends directly or indirectly from Claim 1. The deficiencies of *Han* and *Sagner* with respect to Claim 1 have been discussed in Section III. *Vogelstein* teaches a PCR reaction using two molecular beacons (see Vogelstein at page 9236, describing a molecular beacon (MB)-Green and a MB-Red). In stark contrast, Claim 1 of this Application embodies a system comprising a multi-channel detector to detect **four** or more electromagnetic signals from **four** or more fluorophores. However, the Office Action failed to explain how *Vogelstein*, in combination with *Han* and *Sagner* can arrive at the independent system Claim 1, comprising a processor controlled analyzer to receive, from the multi-channel detector, **a cumulative signal** based on the first, second, third, and fourth electromagnetic signals [from the **first, second, third, and fourth fluorophores**] and apply a decoding matrix to **the cumulative signal** to unambiguously detect the presence or absence of each of M analytes by associating, for each analyte, a first value in a first component of **the cumulative signal** and a second value in a second component of **the cumulative signal**, wherein each first value is an intensity or range of intensities and each second value is a wavelength or a range of wavelengths, and wherein the second values comprise the first, second, third, and fourth wavelengths, and the determination is made without mass spectrometry or melting curve analysis…" Thus, Applicant respectfully request the Examiner to explain how the system of Claim 1 would be obvious in view of the combination of *Han*, *Sagner*, and *Vogelstein*. In the absence of such an explanation supporting a *prima facie* obviousness rejection, Applicant respectfully requests withdrawal of the rejection with respect claims 15-16, which depends from Claim 1.

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Claims 21, 25-26, and 28 depends directly or indirectly from Claim 20. The deficiencies of *Han* and *Sagner* with respect to Claim 20 have been discussed in Section III. The deficiencies of *Vogelstein* in combination with *Han* and *Sagner* have been discussed above. Claim 20, as amended is directed to a reaction mixture for unambiguously detecting a presence or absence of a plurality of non-immobilized polynucleodtide analytes of a single sample, the reaction mixture comprising **four** hybridization probes. The Office Action failed to explain how *Vogelstein*, in combination with *Han* and *Sagner* can arrive at the independent reaction mixture Claim 20. Thus, Applicant respectfully request the Examiner to explain how the reaction mixture of Claim 20 would be obvious in view of the combination of *Han*, *Sagner*, and *Vogelstein*. In the absence of such an explanation supporting a *prima facie* obviousness rejection, Applicant respectfully requests withdrawal of the rejection with respect claims 21, 25-26, and 28, which depends from Claim 20.

V. Nonstatutory Double Patenting

The Examiner further rejected the following claims of this Application alleging nonstatutory double patenting:

1. claim 19 for nonstatutory double patenting over claim 1 of U.S. Patent No. 8,838,394 B2 in view of *Sagner*;

2. claims 1 and 20 for nonstatutory double patenting over claim 1 of U.S. Patent No. 10,068,051 B2 in view of *Sagner*;

3. claim 1, 15, 20, and 21 for nonstatutory double patenting over claim 1 of U.S. Patent No. 10,770,170 B2 in view of *Sagner*; and

4. claims 1 and 20 for nonstatutory double patenting over claim 22 of copending U.S. Application No. 16/937,464 in view of *Sagner*.

Applicant requests that these matters be held in abeyance until such time as the claims of these applications are otherwise allowable. It is believed to be premature to file a Terminal Disclaimer before the scope of the claims has been settled.

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CONCLUSION

In view of the foregoing amendments and remarks, Applicant respectfully requests reconsideration of this application and the timely allowance of the pending claims.

If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided. Please grant any extensions of time required to enter this response and charge any additional required fees to Deposit Account No. 50-4561.

Respectfully submitted,

SHEPPARD MULLIN RICHTER & HAMPTON LLP

Dated: February 1, 2024

By: /David E. Heisey/
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DESCRIPTION CN104271767A

10 Encoding and decoding signals in multiplexed biochemical assays

[0001]

14 cross reference

[0002]

18 This application claims the benefit of U.S. Provisional Application 61/594,480, filed on February 3, 2012, and U.S. Provisional Application 61/703,093, filed on September 19, 2012, each of which is incorporated herein by reference in its entirety.

[0003]

24 background

[0004]

28 Multiplex reactions offer significant advantages over traditional single-pass reactions, including performing multiple reactions in parallel on the same sample, performing more reactions using the same chamber, and extracting from the sample in a fast and efficient manner. The ability to enrich information.

31 However, to realize these advantages, multiplexed assays often require complex reporting mechanisms, i.e., spectrally resolvable fluorescence or chemiluminescence (e.g., PCR, ELISA), spatially resolvable signals (e.g., microarrays, gel electrophoresis).), a temporally resolved signal (e.g., capillary electrophoresis), or a combination thereof (e.g., Sanger sequencing).

35 There is a need for multiplex reactions that can be performed in a single solution.

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[0005]

39 Summary of the invention

[0006]

43 The present disclosure provides methods, compositions, systems and kits for multiplex detection of analytes.

44 In some cases, the present disclosure provides assays that are capable of unambiguously detecting the presence or absence of in a single sample volume without performing immobilization, separation, mass spectrometry, or melting curve analysis. The presence or absence of each of at least 7 analytes in any combination.

47 In some examples, each analyte is encoded as a value for one (or at least one) component of the signal.

[0007]

51 In some examples, the present disclosure provides assays 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 when The maximum cumulative value of a component (eg, intensity) of the signal in the presence of all analytes.

[0008]

57 In some cases, each analyte is encoded as at least one first value in a first component of the signal (e.g., at least one intensity or intensity range) and at least one second value in a second component of the signal (e.g., at least one wavelength or range of wavelengths).

60 In some examples, each analyte is encoded as a first value in the first component of the signal for each of a plurality of second values in the second component of the signal (e.g., at a plurality of wavelengths or signal strength per wavelength range or signal strength range per wavelength range).

[0009]

66 In some examples, assays provided herein are capable of unambiguously detecting the presence or absence of M analytes in any combination of presence or absence, where $M = C*\log2(F+1)$, where C is The number of second values used to encode an analyte, and F is the maximum cumulative value of the first component of the signal for any second value in the presence of all analytes.

[0010]

73 In some cases, the present disclosure provides assays 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 code The number of codes per layer in the solution, and T is the number of layers.

76 In some cases, the number of layers $T = \log 4 (F + 1)$, and F is the maximum cumulative value of the first component of the signal for any second value in the presence of all analytes.

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[0011]

81 In some examples, the first value is intensity or intensity range.

82 In some cases, the coding scheme includes at least three intensities or intensity ranges.

[0012]

86 In some cases, the second value is a wavelength or range of wavelengths.

87 In some examples, the encoding scheme includes at least five wavelengths or wavelength ranges.

[0013]

91 In some embodiments, the signal is an electromagnetic signal.

92 In some cases, the electromagnetic signal is a fluorescent emission signal.

93 In some embodiments, the intensity of the fluorescence emission signal at at least four wavelengths or ranges of wavelengths is measured.

[0014]

98 In some cases, assays provided herein are performed using reagents that are lyophilized prior to use.

[0015]

102 In some cases, the detection step is performed using reagents including hybridization probes.

103 In some examples, the number of hybridization probes is greater than the number of analytes.

104 In some cases, a hybridization probe set includes one or more hybridization probes that are specific for different analytes and include the same fluorophore or combination of fluorophores.

106 In some examples, the sample is contacted with at least 18 of the hybridization probes.

107 In some examples, the detection step is performed using a reagent that includes at least one pair of primers. In some cases, at least one pair of primers is capable of amplifying a region complementary to at least three of said hybridization probes.

[0016]

113 In some examples, the measured signal is generated during a polymerase chain reaction.

114 In some cases, the polymerase chain reaction is selected from the group consisting of: end-point polymerase chain reaction, real-time polymerase chain reaction, digital polymerase chain reaction, and combinations thereof.

[0017]

120 In some examples, at least one cumulative measurement is performed on the solution.

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[0018]

124 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: at least one additional value from the signal values of components, values of at least one component from different signals, and combinations thereof.

128 For example, at least one additional value may be selected from the group consisting of: fluorescence emission intensity, fluorescence emission wavelength, resonance energy transfer (FRET) emission intensity, FRET emission wavelength, electrochemical signal, chemiluminescence wavelength, chemiluminescence intensity, Fluorescent bleaching rate, chemiluminescent bleaching rate, and combinations thereof.

[0019]

137 In some cases, chromatograms were constructed.

138 A chromatogram is constructed by plotting all possible combinations of first and second values for a positive control sample for each analyte.

[0020]

143 In some examples, at least one analyte includes a polynucleotide.

144 In some cases, the polynucleotide is from a source selected from the group consisting of animals, plants, bacteria, fungi, parasites, and viruses.

146 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, mycobacteria, 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 papillomavirus E6, and human papillomavirus E7.

[0021]

154 In some cases, the sample is selected from the group consisting of: clinical samples, food samples, environmental samples, pharmaceutical samples, and samples from consumer products.

[0022]

159 In some examples, information regarding the presence or absence of an analyte is transmitted over a computer network.

[0023]

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164 In some cases, the physician is provided with information regarding the presence or absence of the analyte.
165 In some instances, clinical decisions are made based on this information.

[0024]

169 In some instances, at least one step of the methods provided herein is performed using instructions on a computer-readable medium.
171 In some cases, the instructions are located on a remote server.
172 In some instances, the instructions are located on the thermal cycler.
173 In some cases, the instructions are located on a computer that communicates with the thermal cycler.

[0025]

177 In some cases, at least one analyte is a positive control analyte.

[0026]

181 In some instances, the encoding scheme is non-degenerate.
182 In some cases, encoding schemes are designed to be non-degenerate.
183 In some examples, the degeneracy is eliminated by enumerating every reasonable result that can be obtained from the coding scheme, identifying each reasonable result that is degenerate, and eliminating at least one potential analyte code from the coding scheme. The coding scheme is made non-degenerate.

[0027]

189 In some cases, the assays provided herein are endpoint assays.
190 In some examples, the present disclosure provides assays that end at a threshold number of cycles set by the detection limit of the instrument.

[0028]

195 In some examples, the assays provided herein are liquid phase assays.

[0029]

199 In some cases, the assays provided herein are quantitative.

[0030]

203 In some cases, the present disclosure provides a method of detecting the presence or absence of each of a plurality of analytes, the method comprising: (a) encoding each of said analytes as a first value of a signal, a coding scheme is thereby generated, wherein each said analyte is represented by said first value in said coding

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scheme, wherein said coding is performed in a manner that eliminates degeneracy; (b) providing a provision that includes or may include a sample of at least one of said analytes; (c) contacting said sample with an analyte-specific reagent that produces said first value in the presence of each of said analytes as represented in said coding scheme; (d) cumulatively measuring the first value of the signal in the sample, thereby providing a cumulative measurement; and (e) determining whether each of the analytes is present or present based on the cumulative measurement and the encoding scheme. does not exist.

[0031]

215 In some examples, the methods described in the previous paragraph are 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 when all The maximum cumulative value of the first value in the presence of the analyte.

218 In some cases, the first value is intensity or intensity range.

219 In some examples, the first value has a minimum value in the encoding scheme 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.

221 In some cases, the first value is increased in the encoding scheme by an amount equal to the cumulative maximum of the previous first value plus one.

223 In other cases, the first value is incremented in the encoding scheme by an amount greater than the cumulative maximum of the previous first value plus one.

[0032]

228 In some cases, the present disclosure provides methods of detecting the presence or absence of each of a plurality of analytes, the method comprising: (a) encoding each with at least one first value and at least one second value said analyte, 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, resulting in an encoding scheme wherein each said analyte represented by said at least one first value and said at least one second value in said encoding scheme, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (b) providing includes or may include at least one a sample of said analyte; (c) combining said sample with an analyte represented in said coding scheme that produces said at least one first value and at least one said second value in the presence of each said analyte specific reagent contact; (d) cumulatively measuring the first value and the second value in the sample, thereby providing a cumulative measurement; and (e) based on the cumulative measurement and the encoding scheme, Determining the presence or absence of each of said analytes, wherein when degeneracy is eliminated, the method is capable of unambiguously detecting in a single volume, when each of said reagents produces only one second value. The presence or absence of each of at least 6 analytes in any combination.

[0033]

245 In some cases, each analyte in the method described in the previous paragraph is encoded as a first in a first component of the signal for each of a plurality of second values in the second component of the signal. value.

247 In some cases, when degeneracy is eliminated, the method is able to unambiguously detect the presence or

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absence of each of at least 7 analytes in a single volume using 4 second values.

249 In some examples, when degeneracy is eliminated, the encoding scheme includes T non-degenerate layers, where T = log 4 (F + 1), and F is with respect to any second value when all analytes are present The maximum accumulated value of the first component of the signal. In some cases, when degeneracy is eliminated, a method can unambiguously detect 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 layer. In some examples, when degeneracy is eliminated, a method can unambiguously detect 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 encoding scheme, and F is the maximum cumulative value of the first component of the signal for any second value when all analytes are present.

[0034]

261 The present disclosure also provides non-degenerate encoding schemes that are capable of unambiguously encoding the presence or absence in a single sample volume without performing immobilization, separation, mass spectrometry, or melting curve analysis. The presence or absence of each of at least 7 analytes in any combination.

[0035]

268 In some examples, the non-degenerate coding scheme of the present disclosure is generated by a method including: (a) generating a code for each potential analyte, wherein each potential analyte passes at least one component of the signal encodes at least one value; (b) enumerates every reasonable cumulative result for all possible combinations of each analyte present or absent; (c) identifies every reasonable degenerate result; and (d) Eliminate at least one code to eliminate degeneracy.

273 In some cases, a coding scheme may be expanded by adding additional codes that are at least one unit greater than the sum of all previous codes in at least one component of the signal.

275 In some cases, coding schemes are generated by mathematical iterations where the structure guarantees non-degeneracy.

[0036]

280 In some cases, the present disclosure provides a system for detecting the presence or absence of each of a plurality of analytes, the system comprising: (a) encoding a first value for each of the analytes as a signal, thereby generating a coding scheme, wherein each said analyte is represented by said first value in said coding scheme, wherein said coding is performed in a manner that eliminates degeneracy; (b) providing a method including or a sample that may include at least one of said analytes; (c) combining said sample with an analyte-specific reagent that produces said first value when each said analyte is present, as represented in said coding scheme contact; (d) cumulatively measuring the first value of the signal in the sample, thereby providing a cumulative measurement; and (e) based on the cumulative measurement and the encoding scheme, determining each of the The analyte is present or absent.

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[0037]

292 In some cases, the present disclosure provides a system for detecting the presence or absence of each of a plurality of analytes, the system comprising: (a) encoding with at least one first value and at least one second value for each of said analytes, 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, resulting in an encoding scheme, wherein each of said the analyte is represented by said at least one first value and said at least one second value in said coding scheme, wherein said coding is performed in a manner that reduces or eliminates degeneracy; (b) providing a method that includes or may include at least a sample of said analytes; (c) comparing said sample to said at least one first value and said at least one second value represented in said coding scheme when each said analyte is present. contacting an analyte-specific reagent of the value; (d) cumulatively measuring the first value and the second value in the sample, thereby providing a cumulative measurement; and (e) based on the cumulative measurement and the The encoding scheme determines the presence or absence of each of the analytes, wherein the method is capable of unambiguously detecting the presence or absence of each of the analytes in a single The presence or absence of each of at least 6 analytes in the volume in any combination of presence or absence.

[0038]

309 In some cases, the present disclosure provides methods for detecting the presence or absence of each of a plurality of analytes for a third group, the method comprising: (a) obtaining each of said analytes from the group identification; (b) encoding each of said analytes as a first value of a signal, thereby producing a coding scheme, wherein each of said analytes is represented by said first value in said coding scheme, wherein said coding is performed in a manner that eliminates degeneracy; (c) providing to said party the analyte specificity that yields said first value when each said analyte is present, as represented in said coding scheme a reagent that: (i) contacts a sample that includes or may include at least one of said analytes with said reagent; and (ii) cumulatively measures said first value in said sample, thereby providing a cumulative measurement results; and (d) obtaining said cumulative measurements from said group; (e) determining whether each of said analytes is present or absent based on said cumulative measurements and said coding scheme; and (f) placing Information regarding the presence or absence of each of the analytes is provided to the group.

[0039]

323 In some cases, the present disclosure provides methods for detecting the presence or absence of each of a plurality of analytes for a third group, the method comprising: (a) obtaining each of said analytes from the group identification; (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 the signal, and said second value is a value from the second component of said signal resulting in a coding scheme, wherein each said analyte is represented in said coding scheme by said at least one first value and said at least one second value, wherein said coding is performed in a manner that reduces or eliminates degeneracy; and (c) providing said party with the results of generating said at least one first value in the presence of each of said analytes and said at least one second value of an analyte-specific reagent, said body: (i) contacting a sample that includes or may include at least one said

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analyte with said reagent; and (ii) cumulatively measuring said sample the first value and the second value, thereby providing a cumulative measurement; and (d) obtaining the cumulative measurement from the group; (e) based on the cumulative measurement and the encoding scheme, determining each the presence or absence of each of the analytes; and (f) providing information to the party regarding the presence or absence of each of the analytes.

[0040]

340 In some cases, the present disclosure provides compositions for detecting the presence or absence of each of a plurality of analytes, the compositions comprising analyte-specific reagents, each reagent producing a value including a first A signal in which the reagent is capable of unambiguously detecting, in any combination of presence or absence, at least 7 The presence or absence of each analyte.

[0041]

347 In some cases, the present disclosure provides compositions for detecting the presence or absence of each of a plurality of analytes, the compositions comprising an analyte-specific reagent, each reagent producing a composition including at least one first a signal of one value and at least one second value, the at least one first value being a value from a first component of the signal and the at least one second value being a value from a second component of the signal, wherein , said reagents are capable of detecting the presence of each of at least 6 analytes in any combination of presence or absence in a single volume, when each said reagent produces only one second value, or does not exist.

[0042]

357 In some cases, the present disclosure provides kits for detecting the presence or absence of each of a plurality of analytes, the kits comprising analyte-specific reagents, packaging, and instructions, each reagent Generating a signal that includes a first value, wherein the kit is capable of unambiguously detecting the presence or absence of any analyte in a single sample volume without performing immobilization, separation, mass spectrometry, or melting curve analysis. The presence or absence of each of at least 7 analytes in combination.

[0043]

366 In some examples, the present disclosure provides kits for detecting the presence or absence of each of a plurality of analytes, the kits comprising analyte-specific reagents, packaging, and instructions, each reagent Generating a signal comprising at least one first value from a first component of the signal and at least one second value from a second component of the signal value of a component, wherein the kit is capable of detecting at least 6 analytes in any combination of presence or absence in a single volume when each of said reagents produces only one second value The presence or absence of each analyte.

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[0044]

375 In some cases, the present disclosure provides a kit for detecting the presence or absence of each of a plurality of analytes, the kit comprising a cartridge (601), a vial disposed in the cartridge (601). a snap-in slot, a bottle (602) including a probe for detection of an analyte, a bottle (603) including a primer for amplification, and a bottle (604) including a reagent for amplification, wherein , the kit is capable of unambiguously detecting at least 7 analytes in any combination of presence or absence in a single sample volume without performing immobilization, separation, mass spectrometry, or melting curve analysis. The presence or absence of each analyte in .

[0045]

385 In some examples, the present disclosure provides a kit for detecting the presence or absence of each analyte in a plurality of analytes, including: a box body (601), a snap-in disposed in the box body for placing a bottle. slot, a bottle (602) including a probe for detection of an analyte, a bottle (603) including a primer for amplification, and a bottle (604) including reagents for amplification, each probe Generating a signal comprising at least one first value from a first component of the signal and at least one second value from a second component of the signal Component values, wherein the kit is capable of detecting at least 6 analytes in any combination of presence or absence in a single volume when each of the probes produces only one second value The presence or absence of each analyte.

[0046]

396 Incorporated by reference

[0047]

400 All publications and patent applications mentioned in this specification are hereby 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.

[0048]

406 Brief description of the drawings

[0049]

410 The novel features of the invention are set forth with particularity in the appended claims.

411 A better understanding of the features and advantages of the invention will be obtained by reference to the following detailed description of illustrative embodiments in which the principles of the invention are utilized, and in which are the accompanying drawings:

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[0050]

417 Figure 1 shows a comparison between a conventional encoding method that detects four analytes in four colors, and the encoding method of the present invention that is capable of detecting 16 or more sequences in four colors.

420 Color is indicated by B, G, Y and R, which indicate blue, green, yellow and red respectively.

[0051]

424 Figure 2 shows a schematic representing the detection of 6 analytes (including controls) in four colors.

425 Analytes are detected using hybridization probes attached to fluorophores (B, G, Y, R; blue, green, yellow, and red, respectively) and quenchers (ovals marked with an "X").

427 The control sequence and five other sequences (Dengue = Dengue virus; Tuberculosis = Mycobacterium tuberculosis; P17 = HIV p17; Malaria = Plasmodium falciparum; and Herpes = Herpes simplex virus 2) were used Probe detection labeled with blue fluorophore.

430 Each non-control analyte was detected using 1-3 additional probes.

431 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 an additional probe with a red fluorophore; and so on.

[0052]

437 Figure 3 shows a chromatogram of experimental results obtained as described in Example 2.

[0053]

441 Figure 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.

[0054]

446 Figure 5 shows a schematic diagram of two different methods of detecting the wavelength and intensity of a light signal, such as a fluorescence emission signal.

[0055]

451 Figure 6 shows a schematic diagram of an exemplary kit.

[0056]

455 Figure 7 shows a chromatogram of experimental results obtained as described in Example 3.

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[0057]

459 Figure 8 shows a schematic diagram of reasonable results (top) and unreasonable results (bottom).

[0058]

463 Detailed description of the invention

[0059]

467 Due to several desirable properties, including compatibility with biochemical assays, relatively small size of the fluorescent label, simple means of conjugating molecules of interest, affordability, low toxicity, stability, robustness, inexpensiveness to utilize Due to the detectability of optical systems and the ability to combine with spatial arrays, fluorescence detection has become the preferred technology for multiplex determination.

471 However, standard fluorescence has a broad emission spectrum.

472 Therefore, to avoid spectral overlap (i.e., to maintain spectral resolution) only a relatively small number of colors (e.g., 4 to 6) are typically used simultaneously in multiplexed fluorometry.

[0060]

477 Traditional coding methods for multiplexed fluorometric assays have been to code each analyte with a single color; i.e., $M=N$, where M is the number of detectable analytes and N is the number of spectrally resolvable fluorescent probes.

480 Whenever higher multiplexing factors are required (i.e., $M>N$), fluorescence is often combined with other technical techniques, such as aliquoting, spatial arraying, or sequential processing.

482 These additional processing steps are labor intensive and often require relatively expensive and complex optical and mechanical systems (e.g., spectrometers, mechanized microscope stages, microfluidics, droplet generators, scanners, and similar systems).

485 Such systems are often impractical to configure in certain environments, particularly point-of care and low-resource settings.

487 Therefore, there is a significant need for multiplexed encoding and decoding methods that can provide inexpensive means of multiplexing while avoiding the use of expensive additional processing steps.

[0061]

492 The present disclosure provides methods, systems, compositions, and kits for the detection of multiple analytes in a sample.

494 This article presents methods for detection of analytes based on encoding, analysis, and decoding.

495 In some examples, each analyte to be detected is encoded as a value of a signal (e.g., intensity), where the value is assigned such that the result of the assay unambiguously indicates the presence or absence of the analyte being assayed.

498 In other examples, each analyte to be detected is encoded as a value in each of at least two components of the signal (e.g., intensity and wavelength).

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500 At least two components of the signal can be orthogonal.

501 Similarly, as described more fully elsewhere in this disclosure, multiple orthogonal signals may be used, such as a combination of fluorescent and electrochemical signals.

503 The analyte can be any suitable analyte, such as polynucleotides, proteins, small molecules, lipids, carbohydrates, or mixtures thereof.

505 The signal may be any suitable signal, such as an electromagnetic signal, an optical signal, a fluorescent emission signal, an electrochemical signal, a chemiluminescent signal, and combinations thereof.

507 The at least two components of the signal may be any suitable two components, such as amplitude and frequency, or intensity and wavelength.

[0062]

512 After encoding the analytes, a sample is provided, wherein the sample includes or may include at least one of the encoded analytes.

514 The sample is contacted with one or more analyte-specific reagents specified for each analyte in the coding scheme that produce a specific signal in the presence of the analyte.

516 The reagent may be any suitable reagent capable of generating such a signal in the presence of its corresponding analyte, e.g., an oligonucleotide probe (e.g., a TAQMAN probe) linked to a fluorophore and a quencher.

519 If the reagent is an oligonucleotide probe linked to a fluorophore and a quencher, nucleic acid amplification that produces a signal can be performed.

[0063]

524 After adding reagents, the signal is quantified.

525 In some cases, this 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 value encoding the presence of each analyte, and the cumulative value of the signal. kind of quantitative.

[0064]

531 In some cases, at least two components of the signal (eg, intensity and wavelength) are measured cumulatively over the sample.

533 For example, this measurement can be performed by measuring the intensity at a specific wavelength, or the intensity within a specific wavelength range.

535 The presence or absence of the analyte may then be determined based on the value 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 encoding scheme).

[0065]

541 Coding may be performed in a manner that reduces or eliminates the number of possible degenerate (e.g.,

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ambiguous) results obtained by the method.

543 As described elsewhere in this specification, the complete coding capabilities 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.

546 Likewise, coding schemes can be designed to be non-degenerate so that reduction or elimination of degeneracy is not necessary.

548 A decoding matrix can be constructed to correspond to the constituent signals of the accumulated signal measurements that interpret the accumulated signal measurements (e.g., intensity or intensity at a specific wavelength) as the presence or absence of certain analytes.

[0066]

554 I.

555 definition

[0067]

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

[0068]

564 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.

566 In addition, for the terms "including", "includes", "having", "has", "with", "such as", or variations thereof When used in the specification and/or claims, these terms are not limiting and are intended to be inclusive in a manner similar to the term "comprising".

[0069]

572 The term "about" as used herein generally refers to a range of 15% more or less than 15% from the numerical value specified in the context of the particular usage.

574 For example, "about 10" would include the range from 8.5 to 11.5.

[0070]

578 The terms "dimension" and "component" as used herein, when referring to a signal, generally refer to aspects of the signal that can be quantified.

580 For example, if a signal is generated by a fluorescent molecule, it can be quantified in terms of its wavelength (e.g., first dimension or first component) and its intensity (e.g., second dimension or second component).

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[0071]

585 The term "decoding," as used herein, generally refers to a method of determining which analyte is present based on a cumulative signal and an encoding scheme or decoding capable of converting the cumulative signal into information regarding the presence or absence of one or more analytes. matrix.

[0072]

591 The term "encoding", as used herein, generally refers to the processing of an analyte using a method that includes a value of a signal (e.g., intensity), or a value of each of at least two components of a signal or signals (e.g., wavelength and intensity) codes.

[0073]

597 The terms "oligonucleotide probe linked to a fluorophore and a quencher" and "TAQMAN probe" generally refer to hydrolysis probes used in polynucleotide amplification assays to detect the presence of analytes.

599 These probes include oligonucleotide probes linked to fluorophores and quenchers.

600 As long as the quencher and the fluorophore are in close proximity, the quencher will quench the fluorescence emitted by the fluorophore when excited by the light source.

602 The sequence of the oligonucleotide probe is designed to be complementary to the polynucleotide sequence present in the analyte and, therefore, capable of hybridizing to the polynucleotide sequence present in the analyte.

605 Hybridization of oligonucleotide probes is performed in a nucleic acid amplification reaction (e.g., polymerase chain reaction) including primers.

607 After extension of the primer by DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and quencher into the medium. The proximity between the fluorophore and the quencher is destroyed, and the signal from the fluorophore is no longer quenched. Therefore, 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 the analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be generated.

[0074]

616 The term "orthogonal" as used herein generally refers to at least two components of a signal (e.g., wavelength and intensity) that can vary independently or approximately independently, or at least two distinct signals (e.g., a fluorescence emission signal and a fluorescence emission signal). electrochemical signal).

619 For example, when working with fluorescent molecules, wavelength and intensity are considered to be orthogonal or nearly orthogonal. The wavelength of fluorescent emission will depend on, among other factors, the composition of the fluorescent molecules, and the intensity of the fluorescence will depend on the amount of molecules 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. It is also possible to use components of a signal that vary independently, or multiple signals, as long as the characteristics of the

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components or signals are well enough determined to enable encoding, measurement and decoding. For example, if changes in one component or signal affect changes in another component or signal, both components or signals can still be used as long as the relationship between the changes is understood.

[0075]

632 The terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to a biological molecule that includes a plurality of nucleotides.

634 Exemplary polynucleotides include deoxyribonucleic acid, ribonucleic acid, and synthetic analogs thereof, including peptide nucleic acids.

[0076]

639 The term "probe" as used herein generally refers to an agent capable of generating a signal in the presence of a specific analyte.

641 Probes typically have at least two parts: a part that specifically recognizes the analyte, or a part thereof; and a part that generates a signal in the presence of the analyte, or a part thereof. As described above and elsewhere in this disclosure, the probe can be an oligonucleotide probe linked to a fluorophore and a quencher. A probe can also be any agent that generates a signal in the presence of an analyte, such as an antibody that detects the analyte, with a fluorescent label that emits or is quenched by binding of the antibody to the analyte. Any suitable probe can be used with the methods set forth in this disclosure, as long as the probe produces a quantifiable signal in the presence of the analyte. For example, the analyte-specific portion of the probe can be coupled to an enzyme that converts an uncharged substrate into a charged product in the presence of the analyte, thereby increasing the conductivity in the medium over time. . In this case, different analytes can be encoded in different ratios by coupling the analyte-specific portion of the probe (e.g., hybridization probe or antibody) to the enzyme. The rate at which increased conductivity in the medium is produced will accumulate for all analytes present in the medium. According to the methods provided herein, encoding an analyte is capable of converting conductivity measurements into unambiguous (i.e., non-degenerate) results that provide information regarding the presence or absence of a specific analyte. Similarly, the probe may include an enzyme that produces a chemiluminescent product from a substrate. The amount of chemiluminescence can then be used to encode the presence of a specific analyte.

[0077]

660 II.

661 Encoding and decoding methods

[0078]

665 A.

666 Traditional fluorescence encoding and decoding methods

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[0079]

670 A common method for determining the presence of an analyte is to use four spectrally resolvable fluorescent molecules to indicate the presence or absence of four analytes.

672 The left-hand side of Figure 1 shows an example of this approach. The encoding method is shown on the left-hand side of Figure 1, where the four analytes (Sequence 1, Sequence 2, Sequence 3, and Sequence 4) are each encoded by a single color (blue, green, yellow, and red, respectively). Colors represent fluorophores attached to oligonucleotide probes that also include quenchers. In the system shown on the left hand side of Figure 1, there are four different oligonucleotide probes, each including a single fluorophore (blue, green, yellow, or red) and a quencher. The presence or absence of the analyte is determined based on the presence or absence of signal in a particular color.

[0080]

682 The graph on the left hand side of Figure 1 shows intensity versus color for a hypothetical sample containing two analytes: Sequence 1 and Sequence 3.

684 The presence of these analytes is determined based on measurements of the blue signal (corresponding to sequence 1) and the yellow signal (corresponding to sequence 3). The absence of sequence 2 and sequence 4 is indicated by the absence of blue and red signals.

[0081]

690 Table 1 shows the interpretation of the binary format for this encoding scheme.

691 Each analyte is encoded as a value in each of two components of the fluorescence signal: (1) color (also called wavelength; or wavelength range) and (2) intensity (indicated by numbers in the table) . For example, A (eg, sequence 1) has a color of blue and an intensity of 1; B (eg, sequence 2) has a color of green and an intensity of 1; and so on. Quantify the intensity of the signal within each color range as described herein, for example, by measuring the intensity of the signal within a specific wavelength range as determined by a bandpass filter. A result of 1000 indicates that only analyte A is present; a result of 1100 indicates that both analytes A and B are present; and so on.

[0082]

701 Table 1.

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

[0084]

707 B.

708 Coding methods using more than one color per analyte

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[0085]

712 The traditional methods described above suffer from the fact that they are limited by the number of spectrally resolvable fluorophores.

714 More specifically, the number of detectable analytes is equal to the number of spectrally resolvable fluorophores.

716 Therefore, the number of analytes can only be increased by increasing the number of spectrally resolvable fluorophores.

[0086]

721 The present disclosure provides methods to overcome this limitation.

722 More specifically, in some cases, the methods described herein can be used to detect more than one analyte per fluorophore by utilizing at least two components of the signal during encoding. For example, using the methods provided herein, each fluorophore can detect 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. In some cases, the methods provided herein can 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, per fluorophore. 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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. In some cases, the methods provided herein can be used to detect 1.5-2, 2-4, 1.5-4, 4-6, 2-6, 6-10 analytes per fluorophore.

[0087]

734 In some cases, methods provided by the present disclosure may include the use of contrasting colors.

735 The control color can be connected to one or more probes that bind the positive control analyte and each analyte to be detected in the sample. A single control probe can also be used if the same sequence occurs in the positive control analyte and in each analyte being tested. If the same sequence does not occur in the positive control analyte and in each analyte to be detected, different probes can be used, but each probe can still be connected to the control color.

[0088]

743 For example, building on the traditional approach described above, a color (e.g., blue) can be used to encode the presence of a control analyte that is always present in the sample.

745 Control analytes may be added to the sample or may be inherently present in the sample. The remaining colors (eg, green, yellow, red) can be used to encode the presence of additional analytes. Table 2 shows an example of such an approach.

[0089]

751 Table 2.

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752 Four analytes (including controls) are coded in four colors and up to two colors per analyte.

[0091]

756 In Table 2, the presence of a control group (p) is indicated by a result of 1000.

757 The presence of control and analyte A is indicated by the results of 2001.

758 The presence of the control and all other three analytes is indicated by a result of 4111. The control color (blue) provides an indication of normal operation of the assay. The intensity of the blue color reports the number of analytes present in the test sample. Of course, any color can be used as a contrasting color. One skilled in the art will recognize that certain practical considerations may make it preferable to use one color over another as a contrasting color. For example, if a color is better detected in a particular optical system (or fluorophore system), it may actually be preferable to use that color as a control color.

[0092]

767 The coding scheme shown in Table 2 codes each analyte using a control color and an additional color (up to 2 probes per analyte).

769 However, as shown below, when using up to 4 colors per analyte, the number of analytes that can be encoded increases. Table 3 shows an exemplary coding scheme, where each analyte is coded 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). Each of the other seven analytes is coded by the presence of one to three additional colors. The colors may be comprised of different probes (e.g., oligonucleotide probes linked to a fluorophore and a quencher) or the same probe (which may have multiple fluorophores and multiple quenchers).

[0093]

779 table 3.

780 Eight analytes (including controls) are coded in four colors and up to four colors per analyte.

[0095]

784 In Table 3, the presence of the control and all other seven analytes is indicated by a result of 8444.

785 Three analytes (A, B, and C) are coded by two colors.

786 Three analytes (D, E, and F) are coded by three colors, and one analyte (G) is coded by four colors. The presence of control (p) is indicated by a result of 1000. The presence of control and analyte A is indicated by the results of 2001. The presence of 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, result 2111 has a signal strength of 2X in the blue channel, while result 1000 only has a signal strength of 1X in the blue channel.

[0096]

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794 Using Table 3, each possible cumulative measurement result can be enumerated according to color (blue, green, yellow, red) and intensity (0-8).

796 Table 4 shows the "decoding" generated by enumerating each possible cumulative assay result based on the encoding method shown in Table 3 and providing the corresponding decoding result for each assay based on the analytes present in the sample. matrix". Codes for any of the coding schemes described herein can be generated by enumerating each possible cumulative assay result based on the coding scheme and providing corresponding decoding results for each assay based on the presence (or absence) of the analyte in the sample. Similar decoding matrix. In some cases, one or more analytes may be removed from the coding scheme to reduce or eliminate degeneracy, as described below.

[0097]

806 Table 4.

807 The decoding matrix for the encoding method shown in Table 3.

[0098]

811

[0099]

816

[0100]

821

[0101]

826 The decoding matrix is provided in Table 4 constructed using two assumptions.

827 First, the decoding matrix assumes that the positive control (P) always produces a positive result.

828 Second, the decoding matrix assumes that within each color, regardless of which probe the signal may come from, the intensity is added and measured in the same way as changing the probe concentration.

830 This essentially means that the signal is additive and digital.

831 The conditions on which these assumptions are based can be satisfied by appropriate preparation of the assay. As demonstrated in the examples provided herein, if fluorescent signals are used, the intensity need only be approximately additive and numerical.

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[0102]

837 Table 4 allows cumulative measurements of intensity in four ranges of fluorescence wavelengths (i.e., signal intensity in each color range) to be converted to the corresponding analytes present in the sample.

839 For example, a result of 4321 indicates the presence of p, C, F, and G, and the absence of the other analytes. This result is called a "reasonable" result because it appears in the decoding matrix. In contrast, the result of 4000, although possibly measurable, does not appear in the decoding matrix. More specifically, a result of 4000 cannot be achieved by adding any combination of control codes and analyte codes from Table 3. This result is called an "unreasonable" result. Unreasonable results may indicate an assay failure. Therefore, the control (p) and decoding matrices provide a means of verifying that the assay is functioning properly.

[0103]

848 Table 4 enumerates any combination of fluorophores that produce four resolvable emission spectra (e.g., colors).

850 The term "rank" is used to describe the number of analytes detected including controls. In the example provided above, if the assay is functioning correctly, the rank is equal to the value of the blue signal. For example, a rank of 8 indicates that the control and all other seven analytes (A-G) are present. Rank 2 indicates the presence of a control and only one analyte. The lowest rank is rank 1, which indicates that only controls are present. The number of possibilities at each rank can be enumerated. For example, continuing with reference to the encoding and decoding methods described in Table 3-4, there are 7 possibilities for rank 2. The number of possibilities at rank 3 can be calculated as a combination of 2 out of 7 or 7!/(5! *2!) = 21 possibilities. More generally, the number of possibilities is the combination of N and K, which is N!/((N-K)! *K!). Similarly, at ranks 4, 5, 6, 7 and 8, the numbers of possibilities are 35, 35, 21, 7 and 1 respectively. Reference to Table 4 shows that the table is consistent with theoretical predictions. Therefore, Table 4 is an exhaustive decoding matrix for the encoding methods provided in Table 3.

[0104]

864 C.

865 Reduce or eliminate degeneracy

[0105]

869 The terms "degeneracy" and "degeneracy", as used herein, generally describe situations where a reasonable outcome is ambiguous because it can indicate more than one possibility depending on the presence or absence of the analyte.

872 For example, referring to Table 4, the result 5233 is degenerate because it can be decoded as pADFG or pBDEG. Similarly, the result 4222 is degenerate because it can be decoded into any of the following: pCDG, pAFG, pBEG, pDEF. In contrast, the result 3110 can only indicate pBC and is therefore non-degenerate.

[0106]

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878 The present disclosure provides methods to reduce or eliminate degeneracy, thereby increasing the confidence with which analytes are detected.

880 In one embodiment, the method of eliminating degeneracy includes: (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 the signal the value of analyte codes), wherein eliminating the at least one potential analyte reduces or eliminates degeneracy. For example, with reference to the encoding scheme described in Table 3, and the decoding matrix described in Table 4 (enumerating every reasonable outcome), eliminating any two of analytes D, E, and F eliminates degeneracy. Eliminating any of analytes D, E, and F will not eliminate the degeneracy, but it will reduce it.

[0107]

890 Continuing with the coding scheme described in Table 3, any two of analytes D, E, and F are eliminated from the coding scheme, resulting in a scheme that can analyze 6 analytes (including controls) using only 4 colors without degeneracy. .

893 In contrast, a multiplexed conventional method using 4 colors would only allow reporting of 3 analytes and 1 control. Therefore, the number of analytes that can be analyzed is nearly doubled by using the methods provided herein.

[0108]

899 Figure 2 shows an exemplary embodiment of the invention where 4 colors are used to detect five analytes and one control.

901 Analytes are derived from dengue virus ("Dengue"), Mycobacterium tuberculosis ("TB"), human immunodeficiency virus (HIV) p17 (P17), Plasmodium ("Malaria"), and herpes simplex ("Herpes") nucleic acid. The "color" in this example is a fluorophore attached to an oligonucleotide probe that also includes a quencher. Oligonucleotide probes will usually be different for different analytes but the same color. For example, all probes designated Probe 1 have a blue color but will typically have different oligonucleotide sequences. Of course, the probe designated Probe 1 can also have the same oligonucleotide sequence if complementary sequences are present in each analyte. The mechanism of detection with these probes is described elsewhere in this disclosure. Probe 1 (blue) hybridizes to all 6 analytes including the control. Probe 2 (green) hybridizes analytes from Dengue virus, Mycobacterium, and HIV p17. Probe 3 (yellow) hybridizes analytes from dengue virus and Plasmodium. Probe 4 hybridizes analytes from Dengue virus, Mycobacterium, and Herpes simplex. The coding scheme defined by these probes is shown in Table 5. Referring to Table 3, potential analytes D (1011) and F (1110) have been eliminated from the coding scheme. Therefore, the coding scheme shown in Table 5 is non-degenerate, and each reasonable result from the assay corresponds to the presence or absence of a unique combination of analytes in the sample.

[0109]

918 table 5.

919 Coding schemes for the detection of dengue virus, mycobacteria, HIV, Plasmodium, and herpes simplex are

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illustrated in Figure 2.

[0111]

924 The methods shown above for encoding and decoding, including methods for eliminating degeneracy, apply equally to encoding methods using additional colors.

926 For example, Table 3 could be expanded by including additional colors and additional intensities (described further below).

928 Then as mentioned above, the enumeration can produce a decoding matrix from each reasonable result obtained by the encoding scheme. A decoding matrix similar to the one provided in Table 4 can be constructed for any of the encoding schemes described in this disclosure. Plausible results for degeneracy are then identified, and at least one potential analyte code is eliminated from the coding scheme to reduce or eliminate the degeneracy. Methods of eliminating degeneracy can be performed using software on a computer-readable medium, or using a hardware configuration (eg, a microchip) that performs the method.

[0112]

937 Although degeneracy can be reduced or eliminated by the methods described above, and elsewhere in this disclosure, this disclosure also provides encoding schemes that are non-degenerate by design.

939 For example, after eliminating the degeneracy of the encoding scheme described in Table 3, the encoding scheme can be expanded indefinitely in a non-degenerate manner, where the non-degeneracy is by design (see, e.g., the encoding scheme exemplified in Table 6, below described more fully). Similarly, the present disclosure provides an encoding scheme that is completely non-degenerate by design, and therefore does not require any degeneracy reduction or elimination (see, e.g., the encoding scheme exemplified in Table 8, described more fully below). Therefore, the encoding schemes provided by this disclosure may have reduced or eliminated degeneracy by design, or may be non-degenerate by design.

[0113]

949 D.

950 An encoding method that uses more than one color and more than one intensity

[0114]

954 The encoding method described above (e.g., Tables 3-5) can be further extended by allowing analytes to be encoded by an intensity greater than 1.

956 For example, the coding scheme provided in Table 5 codes each analyte to be coded by a fluorescence intensity of 1 or 0. Allowing higher signal intensity values 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 the control color in order to maintain the analyte counting capability of the control color.

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[0115]

964 Table 6 shows the first three layers of an exemplary encoding scheme utilizing four colors and multiple intensities.

966 Level 1 of Table 6 is a reproduction of Table 3, showing the coding of seven analytes and one control using four colors. As described above, any two of the potential analytes D, E, and F can be eliminated from the encoding scheme to produce a non-degenerate encoding scheme. Level 1 of Table 6 indicates that potential analytes D (1011) and F (1110) have been eliminated from the coding scheme to eliminate degeneracy. Therefore, as described above, the coding scheme shown in Level 1 of Table 6 is able to determine the presence of five analytes and one control using four colors.

[0116]

975 The coding scheme of Layer 1 of Table 6 can be extended to the second layer (Layer 2) by allowing the intensity of any color to be increased.

977 As described above, in order to maintain the sequence counting capability of the control, the intensity of the control color can be maintained at 1. Increasing the intensity of any of the remaining three colors will produce the codes 100Y, 10Y0, 1Y00, 10YY, 1Y0Y, 1YY0 and 1YYY, where Y>1. The minimum value of Y used to encode the new layer is equal to the cumulative maximum value from the existing layer plus one. As described below, it is also possible to use values greater than 1 to maximize the difference between intensities.

[0117]

985 Therefore, in this context, the term "layer" is generally used to describe a group of codes that take full advantage of the encoding capabilities provided by a specific number of first values (eg, intensity) and second values (eg, color) without degeneracy. .

988 For example, Layer 1 of Table 6 takes full advantage of the encoding capabilities provided by four colors with no degeneracy and at most 1 intensity per color. As shown in Table 6, Tier 1, this would result in six coded analytes including controls. Introducing a second layer, as mentioned above, the minimum value of Y can be increased to be equal to the cumulative maximum result from the existing layer plus one (or more than one). In the example provided in Table 6, the intensity of the blue (control) color was kept at 1 to preserve the sequence counting capabilities of this color. Thus, layer 2 includes five non-degenerate coding possibilities by increasing the intensity in the green, yellow and red channels to equal the cumulative maximum result in each of these channels according to layer 1 plus one get. All possibilities for these codes of layer 2 can then be enumerated, and the codes causing degeneracy can be eliminated, or layer 2 can be made by design, using the information used to eliminate the corresponding codes from layer 1. Non-degenerate. Greater coding capacity can then be achieved by adding a third layer, or more layers, built according to a similar approach. The encoding scheme can have an unlimited number of layers, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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 layers.

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[0118]

1005 More specifically, referring to Table 6, analyte H in layer 2 is encoded by 1004.

1006 The value of the control color remains at 1. The value of the red color is equal to 1 plus 1, or 4, based on the cumulative maximum result from the previous layer (3). Likewise, analytes I and J are coded by 1030 and 1400 respectively. The combination of these codes is used to encode the remaining four analytes (K-N), as was done in layer 1 for analytes D-G. This completes layer 2. At level 2, the content of potential analytes K (1034) and M (1430) leads to degeneracy. Therefore, these analytes have been eliminated from the coding scheme to eliminate degeneracy.

[0119]

1015 Continuing with reference to Table 6, the third layer (Layer 3) is constructed using the same principles described above.

1017 Analyte O is coded 1-0-0-16. The value of the control color remains 1. The value of the red color is equal to the cumulative maximum result from the previous layer (15) plus 1, or 16. Likewise, analytes P and Q are encoded by 1-0-9-0 and 1-16-0-0 respectively. A combination of these codes is used to code the remaining four analytes (R-U), as done in Tier 1 for analytes D-G and in Tier 2 for analytes K-N. At level 3, the content of potential analytes R (1-0-9-16) and T (1-16-9-0) leads to degeneracy. Therefore, these analytes have been eliminated from the coding scheme to eliminate degeneracy.

[0120]

1026 The three-tier coding scheme shown in Table 6 shows the coding of 15 analytes and one control using four colors.

1028 This encoding scheme can be expanded indefinitely by adding more intensities to create additional layers and/or adding more colors to create additional encoding capabilities within layers. As described in this disclosure, methods to reduce or eliminate degeneracy can be used when adding each intensity and/or color to reduce or eliminate the results of degeneracy.

[0121]

1035 More generally, the encoding scheme depicted in Table 6 is a non-degenerate, infinite extension of the encoding scheme depicted in Table 3-5.

1037 The maximum intensity measured cumulatively in the first layer is 6. The maximum intensity measured in the second layer of accumulation is 15. The maximum intensity measured cumulatively at level 3 is 63, and so on. Given the maximum cumulative intensity value (F), the maximum number of layers (T) available in this coding scheme is $T = \log 4 (F + 1)$. The coding scheme depicted in Table 6 provides five (P) non-degenerate codes per level. Therefore, the maximum number of codes $M = 5*\text{Log } 4 (F + 1)$, or $M = P * T$, where P is the number of non-degenerate codes per level and T is the number of levels. For example, given F = 63, for 5 non-degenerate codes per level, the maximum number of codes (ie, analytes) is 15. This formula does not include code 1000, which is retained as a positive control in Table 6. To include controls in the total number of analytes, one can simply add one, providing the formula $M = (P*T) + 1$.

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[0122]

1049 The methods provided in this disclosure can be used to scale this encoding scheme infinitely.

1050 For example, by utilizing different combinations of intensity (i.e., first value) and color (i.e., second value), one can change the number of non-degenerate codes per layer (P) and the number of layers (T) , encoding any number (M) of analytes. For example, the number of non-degenerate codes per layer 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 layers 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.

[0123]

1060 Table 6.

1061 Code 15 analytes and 1 control using four colors and multiple intensities.

[0124]

1065

[0125]

1070

[0126]

1075 The right-hand side of Figure 1 shows an illustrative example of the method described above.

1076 More specifically, the right-hand side of Figure 1 shows a coding method in which each of 9 or more analytes (Sequence 1 - Sequence 9, etc.) is represented by at least two colors, and in each color Intensity changes are encoded.

1079 Colors represent fluorophores attached to oligonucleotide probes that also include quenchers.

1080 Systems can be designed such that each probe is labeled with a single fluorophore, or each probe is labeled with more than one fluorophore. For example, the code for analyte H is 1004 in Table 6. An intensity of 4 in the red channel can be achieved by using H-specific probes that include 4 red fluorophores, or by using 4 H-specific probes that each include a single red fluorophore. Of course, any combination of probes and fluorophores that yields a result of 4 in the red channel is equally suitable, such as 2 probes each containing 2 red fluorophores, and 1 probe containing 1 red fluorophore. and 1 probe containing 3 red fluorophores, or just 1 probe containing 1 red fluorophore, but present in the reaction mixture at 4x the amount.

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[0127]

1090 The encoding scheme depicted on the right-hand side of Figure 1 may be as shown in Table 7.

1091 The analysis result shown on the right hand side of Figure 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 sequence 1, sequence 3, sequence 4, and sequence 5.

[0128]

1098 Table 7.

1099 The right-hand side of Figure 1 illustrates the encoding scheme.

[0129]

1103

[0130]

1108 E.

1109 Use one color and one intensity for each analyte, but use different encoding methods for intensities within analytes

[0131]

1114 In some of the methods presented here, each analyte is encoded by a single combination of color and intensity.

1116 For example, in a four-color system, the first four analytes could be coded by 1000, 2000, 4000, and 8000.

1117 The next four analytes can be coded by 0100, 0200, 0400, and 0800. Analytes 9-12 and 13-16 will be distributed similarly as shown in Table 8.

[0132]

1122 Like the encoding method described in Table 6, this encoding scheme is limited by the bandwidth of the instrument used by the construction to be theoretically infinite, non-degenerate, and only used by the measured signal.

1125 However, this encoding scheme is able to quantify more analytes per unit of bandwidth than the encoding method described in Table 6. The reason for this is that the available multiplexing of the signal is used with maximum efficiency, since each intensity level is utilized in the encoding (i.e. there are no gaps in the color spectrum; see description of color spectrum below). Table 8 shows one embodiment of this method, illustrating a four-layer encoding based on four colors and intensities 1, 2, 4 and 8. The coding scheme is

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non-degenerate, and if all 16 analytes are present, the result is 15-15-15-15. In terms of bandwidth utilization, this encoding method is more efficient than the encoding method given above. However, this method does not have the proofreading capabilities of the first solution because all results decoded into reasonable outputs in the first solution have gaps in the color spectrum.

[0133]

1137 Table 8.

1138 An example of an encoding method that uses one color and one intensity for each analyte, but uses different intensities within the analyte.

[0134]

1143

[0135]

1148

[0136]

1153 The methods shown above, and exemplified in Table 8, can be extended by introducing additional colors and/or intensities.

1155 For example, you can use 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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.

1158 Additional intensity levels are also available, such as 16, 32, 64, 128, 255, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, etc.

1160 The method is generalizable and can be used to encode an unlimited number of analytes. Each analyte is represented by a code in a single color, where the value of the code in that single color is equal to the sum of all previous values plus one. For example, if the first code in a particular color contains 1, subsequent codes are 2, 4, 8, 16, 32, 64, 128, and so on. Other progressions are possible but are not considered efficient based on bandwidth usage. However, one of ordinary skill in the art will recognize that when one wishes to maximize the spacing between values, less efficient bandwidth usage may be desired. Similarly, the value 1 may be excluded from the encoding scheme, for example, to maximize the difference in intensity between the first encoded value and the noise of the instrument. For example, in some cases, the coding scheme may start with a value of 2, which would provide the analyte code with progressions of 2, 4, 8, 16, 32, 64, 128, etc., while the progression of the cumulative result would be It's 2, 4, 6, 8, 10, 12, 14, 16, etc. This approach creates uniform gaps in the possible cumulative results, allows for higher tolerance to noise compared to analyte code levels of 1, 2, 4, 8, 16, 32, etc., and This corresponds to the cumulative result series of 1, 2, 3, 4,

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5, 6, 7, 8, etc. In this example, the increased robustness in the measurement comes at the expense of a reduced number of codes available in the fixed bandwidth of multiplexing. For example, if only 63 multipath states can be reliably measured, then a coding scheme starting at 1 provides 7 codes per color, while a coding scheme starting at 2 provides 6 codes per color. If 4 colors are available, the former will provide 28 codes and the latter will provide 24 codes. In summary, both starting signal strength and magnitude (i.e., the difference between intensity values) can be measured to maximize the resolution of the instrument's noise and to maximize the difference between intensity values, thereby enhancing discrimination. ability between different experimental results.

[0137]

1183 The encoding scheme illustrated in Table 8 is non-degenerate by design.

1184 Although the coding scheme illustrated in Table 8 uses both intensity and color to code each of the 16 analytes, it is also possible to code each analyte by simply utilizing intensity. For example, given the 16 analytes (A-P) provided in Table 8, a single color coding scheme encoding all 16 analytes could be assigned the values 1, 2, 4, 8, 16, 32, 64, 128, respectively. 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. This encoding scheme is non-degenerate by design, and the cumulative intensity results can be unambiguously decoded to indicate the presence or absence of analytes A-P. This encoding 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 signal (e.g., signal intensity). By adding a second component to this signal (such as color, as depicted in Table 8), the capacity of this encoding scheme can be increased to M = C * Log 2 (F + 1), where C is the The number of colors used.

[0138]

1197 Any suitable value may be used for C or F, as described throughout this specification.

1198 For example, C can 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 can be at least about 2, 4, 8, 16, 32, 64, 128, 255, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, 131072, 262144, 524288, 1048576, etc. The initial starting value of F and the steps in its series can also vary, as described elsewhere in this disclosure.

[0139]

1206 III.

1207 Analyte

[0140]

1211 The analyte may be any suitable analyte analyzed using the methods and compositions of the present invention, wherein the analyte is capable of interacting with a reagent to produce a measurable signal having at least two components.

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1214 Analytes can be naturally occurring or synthetic. Analytes can be present in samples obtained using any method known in the art. In some cases, a sample may be processed before it is used for analysis of the analyte. The methods and compositions presented in this disclosure can be used in solution phase assays without the need for particles (e.g., beads) or solid supports.

[0141]

1221 In some cases, the analyte can be a polynucleotide, such as DNA, RNA, peptide nucleic acid, and any hybrid thereof, wherein the polynucleotide includes any combination of deoxyribonucleotides and/or ribonucleotides.

1224 Polynucleotides may be single-stranded or double-stranded, or contain both double-stranded or single-stranded sequence portions. Polynucleotides may comprise any combination of nucleotides or bases, including, for example, uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine, isoguanidine Purine and any of its nucleotide derivatives. As used herein, the term "nucleotide" may include nucleotides and nucleosides, as well as nucleosides and nucleotide analogs, and modified nucleotides, including both synthetic and naturally occurring species. . The polynucleotide may be any suitable polynucleotide for which one or more reagents (or probes) as described herein can be generated, 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 (small Cajal body-specific RNA, scaRNA), microRNA (miRNA), double-stranded (dsRNA), ribozyme, riboswitch (riboswitch) or viral RNA. The polynucleotide can be contained in any suitable vector, such as a plasmid, cosmid, fragment, chromosome, or genome.

[0142]

1240 Genomic DNA can be obtained from naturally occurring or genetically modified organisms, or from artificially or synthetically produced genomes.

1242 Individual analytes, including genomic DNA, can be obtained from any source and using any method known in the art. For example, genomic DNA can be isolated with or without amplification. Amplification may include PCR amplification, multiple displacement amplification (MDA), rolling circle amplification, and other amplification methods. Genomic DNA can 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., supra). Polynucleotides can be isolated using other methods known in the art, for example as disclosed in Genome Analysis: A Laboratory Manual Series (Volume I-IV) or Molecular Cloning: A Laboratory Manual. If the isolated polynucleotide is mRNA, it can be reverse transcribed to cDNA using conventional techniques, such as described in Sambrook and Russell, Molecular Cloning: A Laboratory Manual, supra.

[0143]

1255 The analyte can be a protein, polypeptide, lipid, carbohydrate, sugar, small molecule, or any other suitable

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molecule that can be detected by the methods and compositions provided herein.

1257 Analytes can be enzymes or other proteins. The analyte may be a drug or metabolite (e.g., an anticancer drug, a chemotherapeutic drug, an antiviral drug, an antibiotic drug, or a biologic). The analyte can be any molecule such as a cofactor, receptor, receptor ligand, hormone, cytokine, blood factor, antigen, steroid, or antibody.

[0144]

1264 The analyte can be any molecule from any pathogen, such as a virus, bacteria, parasite, fungus, or prion (eg, PrPSc).

1266 Examples of viruses include those from the families Adenoviridae, Flaviviridae, Hepadnaviridae, Herpesviridae, Orthomyxoviridae, Papovaviridae, Paramyxoviridae, Picornaviridae, Polyviridae, Oncoviridae, Retroviridae, Rhabdoviridae, and Togaviridae. Specific examples of viruses include adenovirus, astrovirus, bocavirus, BK virus, coxsackie virus, cytomegalovirus, dengue virus, Ebola virus, enterovirus, Epstein-Barr virus, feline leukemia virus, hepatitis virus, A Hepatitis 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 1 Type, HIV type 2, human papillomavirus (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 Type, 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 Type, HPV type 69, influenza virus, JC virus, Marburg virus, measles virus, mumps virus, Norwalk virus, parvovirus, polio virus, rabies virus, respiratory syncytial virus, retrovirus, rhinovirus, Rotavirus, rubella virus, variola virus, vaccinia virus, West Nile virus and yellow fever virus.

[0145]

1284 Examples of bacteria include those from the following genera: Bordetella, Borrelia, Brucella, Campylobacter, Chlamydia, Clostridium Clostridium, Corynebacterium, Enterococcus, Escherichia, Francisella, Haemophilus, Helicobacter), Legionella, Leptospira, Listeria, Mycobacterium, Mycoplasma, Neisseria, Pseudomonas Pseudomonas, Rickettsia, Salmonella, Shigella, Staphylococcus, Streptococcus, Treponema), Vibrio and Yersinia.

1289 Specific examples of bacteria include Bordetella par apertussis, Bordetella pertussis, Borrelia burgdorferi, Brucella abortus, Brucella canis Brucella canis, Brucella melitensis, Brucella suis, Campylobacter jejuni, Chlamydia pneumoniae, Chlamydia psittaci, Chlamydia trachomatix, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium tetani, Corynebacterium diphtheriae), Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Francisella tularensis, Haemophilus influenzae, Helicobacter pylori , Legionella pneumophila, Leptospira interrogans, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Mycobacterium ulcerans Mycobacterium ulcerans, Mycoplasma pneumoniae, Neisseria gonorrhoeae, Neisseria meningitidis, Pseudomonas aeruginosa, Rickettsia rickettsii), Salmonella choleraesuis, Salmonella dublin, Salmonella enteritidis, Salmonella typhi, Salmonella

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typhimurium, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus agalactiae, Streptococcus pneumoniae, Streptococcus pyogenes), Treponema pallidum, Vibrio cholerae, Yersinia pestis and Yersinia enterocolitica.

[0146]

1304 Examples of parasites include those from the genera Acanthamoeba, Babesia, Balamuthia, Balantidium, Blastocystis Blasocystis), Cryptosporidium, Dientamoeba, Entamoeba, Giardia, Isospora, Leishmania Leishmania, Naegleria, Pediculus, Plasmodium, Rhinosporidium, Sarcocystis, Schistosoma, Toxoplasma, Trichomonas and Trypanosoma.

1308 Specific examples of parasites include Babesia divergens, Babesia bigemina, Babesia equi, Babesia microfti, Babesia ducani), Balamuthia mandrillaris, Balantidium coli, Dientamoeba fragilis, Entamoeba histolytica, Giardia lamblia), Isospora belli, Naegleria fowleri, Pediculus humanus, Plasmodium falciparum, Plasmodium knowlesi, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, Rhinosporidium seeberi, Sarcocystis bovihominis, Sarcocystis suihominis), Schistosoma mansoni, Toxoplasma gondii, Trichomonas vaginalis, Trypanosoma brucei, and Trypansoma cruzi.

[0147]

1317 Examples of fungi include those from the genera Apophysomyces, Aspergillus, Blastomyces, Candida, Cladosporium, Coccidioides (Coddidioides), Cryptococcos, Exserohilum, Fusarium, Histoplasma, Pichia, Pneumocystis, Saccharomyces, Sporothrix, Stachybotrys and Trichophyton.

1320 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, Shen Sporothrix schenckii, Stachybotrys chartarum and Trichophyton mentagrophytes.

[0148]

1327 In some cases, the methods provided in this disclosure can be used to detect any of the analytes described above, or elsewhere in the specification.

1329 In some cases, the methods provided in this disclosure can be used to detect panels of analytes described above, or elsewhere in the specification. For example, the combination may include 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, An analyte from the group consisting of 48, 49, 50, 60, 70, 80, 90, 100 or more of the analytes described above or elsewhere in the specification.

[0149]

1337 Analytes can be obtained from any suitable location, including from organisms, intact cells, cell preparations, and cell-free compositions from any organism, tissue, cell, or environment.

1339 Analytes may be obtained from environmental samples, biopsies, aspirates, formalin-fixed embedded tissue,

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air, agricultural samples, soil samples, petroleum samples, water samples, or dust samples. In some cases, analytes can be obtained from body fluids, which may include blood, urine, feces, serum, lymph, saliva, mucosal secretions, sweat, central nervous system fluids, vaginal fluids, or semen. Analytes can also be obtained from manufactured products, such as cosmetics, foods, personal care products, etc. Analytes can be the product of experimental procedures, including recombinant cloning, polynucleotide amplification, polymerase chain reaction (PCR) amplification, purification methods (such as purification of genomic DNA or RNA), and synthesis reactions.

[0150]

1350 In each multiplex assay, more than one type of analyte can be detected.

1351 For example, polynucleotides, proteins, peptides, lipids, carbohydrates, sugars, small molecules, or any other suitable molecules can be detected simultaneously in the same multiplex assay with appropriate reagents. Any combination of analytes can be detected at the same time.

[0151]

1357 Detection of analytes can be useful for any suitable application, including scientific, clinical, diagnostic, prognostic, forensic and monitoring applications.

1359 Typical applications include detection of genetic diseases, identification of genetic fingerprints, diagnosis of infectious diseases, gene cloning, paternity testing, criminal identification, phylogeny, bio-anti-terrorism, environmental monitoring, and DNA computing. For example, an analyte may be indicative of a disease or condition. Analytes can be used to make treatment decisions, or to assess disease status. The presence of the analyte can be indicative of infection with a specific pathogen or any other disease such as cancer, autoimmune disease, cardiopulmonary disease, liver disease, digestive tract disease, etc. The methods provided here can therefore be used to make a diagnosis and make clinical decisions based on that diagnosis. For example, results indicating the presence of bacterial polynucleotides in a sample collected from a subject may result in treatment of the subject with antibiotics.

[0152]

1371 In some cases, the methods and compositions of the invention can 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.

1375 In some cases, the methods and compositions of the invention can be used to detect 7-50, 8-40, 9-30, 10-20, 10-15, 8-12, or 7-12 analytes.

[0153]

1380 In some cases, the present disclosure provides the ability to unambiguously detect any combination of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, in the presence or absence in a single sample volume. 13, 14, 15, 16, 17, 18, 19, 20,

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21, 22, 23, 24, 25, 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, the presence of each of 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, or Absence is determined without the need for immobilization, separation, mass spectrometry, or melting curve analysis.

1386 In some cases, the present disclosure provides the ability to unambiguously detect at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 in any combination of presence or absence in a single sample volume ,13,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28,29,30, 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 The presence of each of , 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes or absence of assay without immobilization, separation, mass spectrometry, or melting curve analysis. In some cases, the present disclosure provides the ability to unambiguously detect less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, in any combination of presence or absence in a single sample volume. 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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, Determination of the presence or absence of 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes No immobilization, separation, mass spectrometry, or melting curve analysis is required.

[0154]

1401 IV.

1402 Signal

[0155]

1406 The methods presented in this disclosure can be used with any quantifiable signal.

1407 In some cases, the present disclosure provides encoding schemes and methods that non-degenerately encode an unlimited number of targets using a single component of a signal (e.g., intensity). For example, as described above and in Example 3, the encoding scheme may rely on multiplexing of signal strength regardless of color. Although fluorescent probes have been used to illustrate this principle, as described elsewhere in this disclosure, the encoding scheme is equally suitable for any other method that provides a quantifiable signal, including electrochemical signals and chemical signals. Glow signal.

[0156]

1416 The methods proposed by the present disclosure may also utilize measurements of a signal in at least two dimensions, also referred to as measurements of at least two components of the signal.

1418 For example, utilizing at least two components of a signal (e.g., color and intensity) allows for more differences per unit of signal intensity bandwidth than the encoding scheme described in the paragraph above that relies on signal intensity to distinguish analytes. code. When at least two components of the signal are utilized, cumulative measurements of at least two components of a single sample volume can be obtained. For example, in the encoding scheme described in Table 5, the presence of each analyte results in a specific intensity (one component of the signal) in each of 4 colors (the second component of the signal).

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The combination of these constituent signals results in a cumulative signal that can be measured by measuring the intensity at each wavelength or range of wavelengths. The cumulative measurements can then be converted into a determination of the presence or absence of the analyte using a corresponding encoding scheme or decoding matrix.

[0157]

1431 In some cases, a quantifiable signal includes a waveform having both frequency (wavelength) and amplitude (intensity).

1433 The signal may be an electromagnetic signal. The 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, the electromagnetic signal may be a fluorescent signal, such as a fluorescence emission spectrum that may be characterized in terms of wavelength and intensity.

[0158]

1440 In certain portions of this disclosure, signals are described and exemplified in terms of fluorescent signals.

1441 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 fluorescent signals also apply to other signals. For example, like fluorescence signals, any of the electromagnetic signals described above can be characterized in terms of wavelength and intensity. The wavelength of a fluorescent signal can also be described in terms of color. Color can be determined based on measuring intensity at a specific wavelength or range of wavelengths, for example, by determining the intensity distribution of fluorescence at different wavelengths, and/or by utilizing a bandpass filter to determine the intensity of fluorescence within a specific wavelength range. Such bandpass filters are commonly used in a variety of laboratory instruments, including quantitative PCR machines. Intensity can be measured with a photodetector. Many wavelengths may be called "channels."

[0159]

1453 In some cases, the methods provided in this disclosure can be used with any signal where the cumulative signal is measured by the constituent signals of the same color, frequency, absorption band, etc.

1455 However, the accumulated signal need not be digital or scale linearly with the number and strength of the constituent signals. For example, if the physical principle of measurement is absorption, then due to the exponential nature of the Beer-Lambert law, the cumulative attenuation is the product of the constituent attenuations and the constituent concentrations are additive. The logarithm of the cumulative attenuation will then scale linearly with the constituent concentration at each absorption band (corresponding to color if fluorescence detection is used). Therefore, the method of the present invention is applicable. The method of the present invention can also be used for chemiluminescent signals and electrochemical signals.

[0160]

1465 The number of signals, and the number of dimensions or components measured, may also be expanded

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beyond that shown in the exemplary embodiments of the present invention, resulting in an expansion of the multiplexing capabilities.

1468 Exemplary embodiments provided in this disclosure utilize encoding schemes constructed using fluorescent signals that have 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 fluorescent signals with electrochemical signals or FRET signals (fluorescence resonance energy transfer).

[0161]

1477 In some cases, more than two components of the signal can be measured.

1478 For example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, 20 or more components of the 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 the signal may be measured. At least 2, but less than 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, or 20 components of the 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 the signal may be measured. These additional components may include kinetic components such as the rate of signal decay and the rate of photobleaching.

[0162]

1487 If fluorescent signals are used, the number of analytes that can be encoded can be further expanded by utilizing additional fluorophores.

1489 For example, you can use 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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. 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. In some cases, less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 may be used ,23,24,25, 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. 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 can be used , or 10-20 fluorophores.

[0163]

1499 Typically, the number of analytes that can be encoded can be further expanded by utilizing an additional first value (e.g., intensity) from the value or range of values of the first component of the signal.

1501 For example, you can use 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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 from the value or range of values of the first component of the signal. 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 from a value or range of values of the first component of the signal. In some cases, less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 may be used ,22,23,24,25,26,27,28, 29,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44, 45,46 , 47, 48, 49,

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or 50 first values from the value or range of values of the first component of the signal. 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 can be used, 10-15, or 10-20 from the value or range of values of the first component of the signal.

[0164]

1514 In examples where intensity is a component of the signal being quantified, such as when utilizing a fluorescent signal, the presence of the analyte may be encoded using multiple intensities or intensity ranges.

1516 For example, the encoding 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 intensity ranges. Coding schemes may utilize at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 22, 23, 24, 25, 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 intensity ranges. Encoding schemes can utilize less 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 intensity ranges. In some cases, encoding schemes 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 intensity ranges.

[0165]

1528 The number of analytes that can be encoded can be further expanded by utilizing an additional second value (e.g., wavelength) from the value or range of values of the second component of the signal.

1530 For example, you can use 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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 from the value or range of values of the second component of the signal. 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 from a value or range of values of the second component of the signal. In some cases, less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 may be used, 22, 23, 24, 25, 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 from a value or range of values of the second component of the signal. 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 can be used, 10-15, or 10-20 second values from the value or range of values of the second component of the signal.

[0166]

1543 In instances where wavelength is a component of the signal being quantified, such as when utilizing a fluorescence signal, the presence of the analyte can be encoded using multiple wavelengths or wavelength ranges.

1546 For example, you can use 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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. 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,

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or more wavelengths or ranges of wavelengths. In some cases, less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 may be used ,22,23,24,25,26,27, 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 wavelength ranges. 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 can be used , 10-15, or 10-20 wavelength or range of wavelengths.

[0167]

1557 In some cases, when degeneracy is eliminated, the methods of the present invention are capable of detecting 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, The presence or absence of 44, 45, 46, 47, 48, 49, 50, or more analytes when each reagent used to generate a signal in the volume produces only one second value (e.g., each reagent , when only one wavelength of light is emitted).

[0168]

1565 In other cases, when degeneracy is eliminated, the method of the present invention is capable of detecting 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, The presence or absence of 45, 46, 47, 48, 49, 50, or more analytes, using a total of four second values in the volume (e.g., a total of four wavelengths or ranges of wavelengths that may Implemented by using four spectrally resolvable fluorophores).

[0169]

1573 As described throughout the specification, the assays provided herein utilize cumulative measurements of samples.

1575 A cumulative measurement may be, for example, a single measurement of an intensity value, or a measurement of intensity values at one or more wavelengths or ranges of wavelengths.

1577 Multiple cumulative measurement results can be obtained.

1578 For example, intensity can be in the range 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, or more wavelengths or wavelength range measurements. Intensity can be 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 wavelength range measurements. Intensity can be 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 wavelength range measurements.

[0170]

1589 More generally, cumulative measurements can be obtained with respect to any quantifiable component of the signal, and with respect to any quantifiable component of the signal at another quantifiable component of the signal.

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1592 For example, at least one first component of the signal may be 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 component measurements of the signal. The at least one first component of the signal may be 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, Second component measurement of 46, 47, 48, 49, 50, or more signals. At least one first component of the signal may be present in 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 component measurements of the signal.

[0171]

1604 As apparent from this disclosure, each analyte to be detected may be encoded as a suitable component utilizing any number of signals, or as a code for any number of signals.

1606 For example, each analyte to be detected could be coded 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 components of the signal or in the signal. 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 the signal or in the signal. In some cases, each analyte to be detected may be encoded in 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 components of or in the signal. 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 can be used A component or signal of , 5-10, 10-15, or 10-20 encodes each analyte. In the encoding scheme each analyte can be encoded by the same number of components of the signal, or signals, or by a different number of components of the signal, or signals.

[0172]

1620 A.

1621 Probes, primers, fluorophores, and quenchers

[0173]

1625 Some methods provided in this disclosure utilize reagents that generate a signal in the presence of an analyte.

1626 Any suitable reagent can be used in the present invention. Typically, the 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 called probes. The probe may be a hybridization probe. The hybridization probe can be an oligonucleotide probe linked to a fluorophore and a quencher (eg, TAQMAN® probe).

[0174]

1633 Methods of the invention may use one or more reagents or probes to detect the presence or absence of each

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analyte.

1635 For example, you can use 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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 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 to detect the presence or absence of each analyte. In some cases, less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 may be used ,23,24,25,26,27, 28,29,30,31,32,33,34,35,36,37,38,39,40,41,42,43, 44,45,46,47 , 48, 49, 50 probes 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.

[0175]

1647 In some cases, samples were associated 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 are contacted to detect the presence or absence of all analytes.

1650 In some cases, the sample is associated 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 probe contacts are made to detect the presence or absence of all analytes. In some cases, samples were associated with less 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 probe contacts to detect the presence or absence of all analytes. In some cases, the number of probes to which the sample is exposed 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.

[0176]

1660 As described above, oligonucleotide probes linked to fluorophores and quenchers can be used to detect the presence of analytes in polynucleotide amplification assays.

1662 As long as the quencher and the fluorophore are in proximity, the quencher quenches the fluorescence emitted by the fluorophore when excited by the light source. The sequence of the oligonucleotide probe is designed to be complementary to the polynucleotide sequence present in the analyte and, therefore, capable of hybridizing to the polynucleotide sequence present in the analyte. Hybridization of oligonucleotide probes can be performed in a nucleic acid amplification reaction (e.g., polymerase chain reaction) involving primers. After extension of the primer by DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and quencher into the medium. The proximity between the fluorophore and the quencher is destroyed, and the signal from the fluorophore is no longer quenched. Therefore, 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 the analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be generated.

[0177]

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1676 Oligonucleotide probes can have one or more fluorophores and quenchers per probe.

1677 For example, in some embodiments, oligonucleotide probes may include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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. Oligonucleotide probes may include 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. Oligonucleotide probes may include less 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.

[0178]

1687 Oligonucleotide probes may include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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.

1690 Oligonucleotide probes may include 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. Oligonucleotide probes may include less 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.

[0179]

1698 Ligation of the probe and quencher to the probe can be performed in the same reaction, or in a series of reactions.

1700 A series of reactions can be performed to label the probe with at least one fluorophore, and the reaction products can be mixed to create a mixture of probes with different fluorophores.

[0180]

1705 If the oligonucleotide probe includes two or more fluorophores, these fluorophores can be arranged to allow (fluorescence) resonance energy transfer (FRET) to occur between the fluorophores.

1709 Simply put, FRET is a mechanism of energy transfer between fluorophores. The use of FRET-based probes allows a single excitation source to produce two distinct fluorescence emission signals through excitation of a single fluorophore. For example, the methods described herein can use paired probes, where one probe in the pair is attached to a fluorophore and a quencher, and the second probe is attached to both fluorophores (close enough for FRET to occur) and Quencher. Any combination of fluorophores and quenchers provided for FRET can be used. This approach doubles the number of fluorescent probes that can be used with an excitation source because a single excitation source can be used to generate two signals: one from a probe with one fluorophore and one from a probe with a fluorophore close enough for FRET Probes that occur with two fluorophores. For example, using the encoding method described in Table 8, the number of

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unambiguously detectable analytes can be increased from 16 to 32 by pairing each probe in Table 8 with a corresponding FRET probe.

[0181]

1723 Primers can be specific for a particular analyte and can amplify a region complementary to the probe.

1724 In some cases, the number of primer pairs used is equal 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, the sample to be analyzed may be associated 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 primer pair contacts. In some cases, the sample to be analyzed may be associated 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, Paired contacts of 45, 46, 47, 48, 49, 50, or more primers. In some cases, the samples to be analyzed may be associated with less 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 primer pair contacts. In some cases, the number of primer pairs is 2-10, 3-15, 4-20, 3-10, 4-10, 5-10, 6-8, or 6-10.

[0182]

1738 Primers can amplify regions of the polynucleotide to which different numbers of hybridization probes hybridize.

1740 For example, at least one pair of primers can amplify genes with 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 regions complementary to hybridization probes. In some cases, all of said pairs of primers can amplify compounds with 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 regions complementary to the hybridization probe.

[0183]

1749 In one example, the first probe can be labeled with the fluorophore 5'-fluorescein amide (5'-FAM) and the quencher black hole quencher 1 (BHQ-1).

1751 The second probe can be labeled with 5'-FAM in close proximity to (e.g., attached to) cyanine 5.5 (Cy5.5) and the quencher black hole quencher 3 (BHQ-3). Upon digestion of the nuclease activity probe via a polymerase, two fluorescent signals are generated from a single excitation wavelength (eg, 470 nm). The fluorophore from the first probe fluoresces at approximately 520 nm. The fluorophore on the second probe is subjected to FRET. The donor fluorophore FAM is excited by an excitation light source (e.g., 470 nm) and transfers its energy to the acceptor fluorophore Cy5.5. The acceptor fluorophore emits at approximately 705 nm. These fluorophores and quenchers are exemplary only. Any fluorophore capable of undergoing FRET, and any quencher that can quench fluorescence, is suitable for use in the present invention. Methods for producing FRET-based probes are described in Jothikumar et al., BioTechniques, 2009, pages

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46(7):519-524.

[0184]

1764 In some embodiments, a single hybridization probe can be used for each analyte.

1765 To take advantage of multi-color coding (e.g., as shown in Table 3), each probe can be labeled with multiple fluorophores in predetermined ratios. The ratio can be determined so that the 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 cheaper to deploy than approaches utilizing multiple probes. Furthermore, in the case of hybridization probes, it is easier to match the sequence of the analyte with one hybridization probe than with multiple probes.

[0185]

1774 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 will work equally well with the invention described in this disclosure. good.

1777 For example, binding molecules specific for the analyte can be used as probes. Non-limiting exemplary binding molecules include antibodies that recognize an analyte and generate a signal in the presence of the analyte.

[0186]

1783 In embodiments of the invention utilizing fluorescent labels, any suitable fluorescent label may be used.

1784 Exemplary fluorescent labels suitable for use in the present invention include rhodamine, parametaminophen, fluorescein, thiofluorescein, aminofluorescein, carboxyfluorescein, chlorofluorescein, methylfluorescein, sulfonate fluorescein, aminorhodol, carboxyrhodol, chlororhodol, methylrhodol, sulforhodol), aminorhodamine, carboxyrhodamine, chlororhodamine, methylrhodamine, sulforhodamine, thiorhodamine, cyanine), indolecarbocyanine, 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, diaminoacridine (proflavin), acridine orange, acridine yellow, arylmethine) derivatives, auramine, crystal violet, malachite green, tetrapyrrole derivatives, porphines, phthalocyanine pigments and bilirubin. Exemplary quenchers include black hole quenchers, such as BHQ-0, BHQ-1, BHQ-2, BHQ-3; ATTO quenchers, such as ATTO 540Q, ATTO 580Q, and ATTO 612Q; and so on.

[0187]

1799 Fluorophores that can be used with the present invention are not limited to any fluorophores described herein.

1801 For example, fluorophores with improved properties continue to be developed, and these fluorophores can

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be readily used with the methods provided by this disclosure. This improved fluorophore includes quantum dots that can 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 required, but many different signals can be quantified, e.g. based on color and intensity. Furthermore, fluorophores with narrow emission spectra would be particularly useful for the methods described here, as such fluorophores can be included in multiplex assays with little or no overlap between their emission spectra, thus providing more "color" and significantly increase the overall number of coded analytes.

[0188]

1812 In some cases, one or more reagents are lyophilized.

1813 Any suitable reagent can be lyophilized. For example, probes, primers, enzymes, antibodies, or any other reagents used for detection can be lyophilized. In some cases, samples including analytes 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 cannot be reliably accessed. In one example, lyophilized reagents include any probe or analyte-specific reagent described in this disclosure or otherwise suitable for use in the present invention. In another example, the lyophilized reagents include PCR primers. In yet another example, lyophilized reagents include reagents suitable for performing PCR reactions.

[0189]

1824 V.

1825 Analytical techniques and instruments

[0190]

1829 The methods provided in this article are suitable for use with various detection methods.

1830 For example, applicable methods use analytical techniques that measure the wavelength and intensity of a fluorescent signal. This can be accomplished by measuring the intensity of the signal across the entire spectrum of wavelengths, or by using a bandpass filter that limits light at certain wavelengths, allowing only certain wavelengths of light to reach the photodetector. Many real-time PCR and quantitative PCR instruments include an excitation light source and a band-pass filter capable of detecting fluorescent signals in four colors (e.g., blue, green, yellow, and red). Therefore, the method of the present invention can be easily applied using instruments widely used in the art. Importantly, the methods and compositions provided herein can be used to detect multiple analytes by obtaining cumulative measurements of a single solution. There is no need to separate. The present invention does not require the use of beads or solid phases. Of course, one of ordinary skill in the art will understand that the present invention can be used in conjunction with separation, beads, or solid phases if desired.

[0191]

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1844 Figure 5 shows two examples of configurations of instruments that can be used to obtain cumulative measurements useful for the method of the invention.

1846 Referring to Figure 5, an instrument is shown at 501 with a detector (e.g., a fluorometer) configured to detect both wavelength and intensity. Referring to 501, at least one excitation light source 502 is directed into a chamber 503 containing an analyte and a reagent that generates a signal in the presence of the analyte 504. As described elsewhere, the analyte can be a nucleic acid, and the signal-generating reagent can be a hybridization probe including a fluorophore and a quencher. If the analyte is present, an emission signal 505 is generated. In the configuration shown at 501, the wavelength and intensity of the emission signal are measured across the spectrum by a detector capable of producing a fluorescence emission spectrum 506.

[0192]

1856 Wavelength and intensity can also be determined using a combination of photodetectors and bandpass filters.

1858 This configuration is used in several thermal cyclers known in the art. Referring to Figure 5, an instrument with a bandpass filter and spot detector is depicted at 507. Referring to 507, at least one excitation light source 508 is directed into a chamber 509 containing an analyte and a reagent that generates a signal in the presence of the analyte 510. As described elsewhere, the analyte can be a nucleic acid, and the signal-generating reagent can be a hybridization probe including a fluorophore and a quencher. If the analyte is present, an emission signal 511 is generated. In the configuration shown at 507, bandpass filters 512, 513, and 514 are used to limit the passage of light to emit light within a specific wavelength range. For example, bandpass filter 512 may limit the passage of light to emit light within wavelength range 1. Bandpass filter 513 can limit the passage of light to emit light in wavelength range 2. Bandpass filter 514 may limit the passage of light to emit light within wavelength range 3. Any number of bandpass 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 in the present invention. Furthermore, the location of the bandpass filter in 507 is not meant to be limiting. In some cases, the emitted light may pass through more than one bandpass filter at any time. In other cases, the bandpass filters may be configured such that all emitted light passes through only one bandpass filter at a time. After passing through the bandpass filter, the filtered light 515, 516 and 517 is detected by the 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 filtered light 515, 516, and 517 can then be quantified by photodetector 518.

[0193]

1879 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.

1882 Digital PCR methods (e.g., droplet digital (BIORAD) and dynamic array (FLUIDIGM)) produce highly sensitive quantification of polynucleotide copy number. The methods presented here can be easily integrated into these systems, significantly expanding their working capabilities by allowing multiplexing in microdroplets or dynamic arrays.

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[0194]

1889 Similarly, the methods described in this disclosure can be applied in real-time PCR assays.

1890 For example, as a PCR run is completed, real-time data can be fully logged. The endpoint value can then be decoded to indicate the presence or absence of the analyte. The value of each cycle threshold (Ct) can be analyzed for each color by detecting the maximum value of the second derivative of the real-time curve for that color. As a specific analyte is amplified, the fluorophore is released from its corresponding probe in the same proportion as the color diversity encoded by that sequence. So a code like 1100 will have the same Ct in blue and green, while a 1400 code will have a green Ct 2 cycles ahead of its blue Ct. This additional data enables determination of the identity and starting amount of each analyte.

[0195]

1900 The method described here can also be used directly on tissue samples.

1901 For example, a tissue sample can be obtained and wells can be created directly in the tissue sample using photolithography. Tissue samples can be fixed beforehand to create wells. The tissue sample can then be analyzed by dispensing appropriate reagents into the wells in the tissue sample. The encoding and decoding methods described in this disclosure can be used for multiplexed detection of analytes etched into wells in tissue. Each well can correspond to a single cell or several cells, providing excellent spatial resolution when performing analyte analysis on different parts of the tissue. This method can be used to detect analytes in different regions of the same tissue.

[0196]

1911 In some cases, the instrument may be modified or constructed, for example, to provide additional excitation light sources at multiple wavelengths, and/or to provide additional bandpass filters or the ability to determine the complete spectrum.

1914 Inclusion of additional excitation sources will allow excitation of a larger number of various fluorophores. Including additional bandpass filters, or modifying or constructing the instrument to determine the entire spectrum, allows detection of a greater variety of emissions from the fluorophore. These techniques can be used to increase the number of fluorophores that can be used in the methods described in this disclosure, and thus the number of analytes that can be detected simultaneously.

[0197]

1922 In some cases, methods described in this disclosure utilize end-point PCR methods.

1923 However, in some cases, results may be obtained before the endpoint. This may be advantageous, for example, when results are needed as quickly as possible. For example, one performs a calibration experiment in which different starting concentrations of the analyte are analyzed to determine the number of cycles at which the signal reaches saturation as a function of the starting amount of analyte. Using this data, a computer that monitors a PCR reaction in real time can be programmed to search for saturation at a

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maximum number of cycles based on the limits of the limit of detection (LOD) of a particular system. If the analyte does not amplify by the maximum cycle number (e.g., except for a possible positive control), then the result is negative for that analyte. If there is amplification of the analyte by the maximum cycle number, then the result is positive for that analyte, and the saturation intensity in each color can be used to decode the result using the encoding scheme. In both cases, there is no need to run the PCR reaction for more than the number of cycles set by the LOD for the instrument. Although PCR methods have been used to illustrate this quantitative method, one of ordinary skill in the art will readily recognize that similar principles can be applied in any catalytic system where the starting amount of analyte limits the reaction rate, and the reaction Rate can be measured and reported by the evolution of signal strength over time.

[0198]

1940 VI.

1941 Compositions and kits

[0199]

1945 The present disclosure also provides compositions and kits for use with the methods described herein.

1946 The compositions can include any of the ingredients, reaction mixtures, and/or intermediates described herein, and in any combination. For example, the present disclosure provides detection reagents for use with the methods provided herein. Any suitable detection reagent may be provided, including hybridization probes labeled with fluorophores and quenchers and primers as described elsewhere in this specification.

[0200]

1953 In some cases, the composition includes a reagent for detecting at least seven analytes using four fluorophores.

1955 In some cases, the composition includes using four fluorophores to detect 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. In some cases, the composition includes using five fluorophores to detect 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 analyte reagents. In some cases, the composition includes using six fluorophores to detect 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 reagents for 50 analytes. In some cases, the composition includes using seven fluorophores to detect 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 Reagents for 50 analytes.

[0201]

1968 In some cases, the composition includes primers.

1969 The composition may include 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

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,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38, 39,40,41,42,43,44,45,46,47,48 , 49, or 50 primer pairs.

[0202]

1974 The invention also provides kits for carrying out the methods of the invention.

1975 Therefore, various kits are provided in suitable packaging. A kit 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 of a plurality of analytes. The kit may include a coding scheme, or decoding matrix, to assist the user in converting cumulative measurements into results indicating the presence or absence of each of a plurality of analytes. The kit may be a diagnostic kit, e.g., a diagnostic kit suitable for the detection of any analyte including the analytes enumerated herein. Kits may contain any of the compositions provided in this disclosure, including those enumerated above.

[0203]

1985 Figure 6 shows a schematic diagram of an exemplary kit of the invention.

1986 Referring to Figure 6, a kit is shown that includes a cartridge 601 containing reagents 602 for detection of an analyte (eg, a polynucleotide probe including a fluorophore and a quencher), and reagents 602 for nucleic acids. Amplification primer 603. The kit may also contain any other reagents, such as reagents 604 suitable for performing an amplification reaction. In the embodiment depicted at 601, the reagents and primers for detection of the analyte are each contained in a single volume (such as a tube or bottle). However, these reagents may also be provided in separate volumes, as depicted by the box shown in 605. Box 605 contains seven separate volumes. Three volumes (606, 607 and 608) contain reagents (or mixtures of such reagents) for detection of analytes, and three volumes (609, 610 and 611) contain primers (or mixtures of primers). The kit may also contain reagents 604 suitable for performing amplification reactions.

[0204]

1998 Figure 6 is provided for illustration purposes only.

1999 Any number of reagents for detection of analytes (e.g., probes), and any number of primers can be included in a single tube or bottle, making it suitable 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 may be included in a single bottle , 19,20,21,22, 23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38, 39,40,41,42,43 , 44, 45, 46, 47, 48, 49, 50, or more reagents for detection of analytes. In other cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, may be included in a single bottle or tube. 19, 20, 21, 22, 23, 24, 25, 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. In some cases, primers and probes may be provided in the same bottle or tube. For example, each primer pair can provide 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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. In other cases, each probe pair can provide 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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. The kit 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,

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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 separate bottles or tubes, or, for example, in the bottle or tube 604 that provides the reagents for the amplification reaction.

2016 Any reagents in the kit can be lyophilized as described elsewhere in this specification.

[0205]

2020 VII.

2021 Systems and software

[0206]

2025 The methods of the present disclosure may also be performed as part of a system.

2026 For example, one or more steps of the methods described herein may be performed by software embodied in a computer-readable medium. Software can be used to encode analytes, check degeneracy, reduce or eliminate degeneracy, and/or generate decoding matrices. Software can also be used to convert cumulative measurements made on an instrument (e.g., a PCR machine) into information about the presence or absence of an analyte. For example, referring to Figure 4, computer 401 may be connected to an input device 403, a display 402, and a printer 404. The computer may perform one or more steps of any of the methods described in this disclosure, including, for example, the encoding method 410, the analysis method 420, and the decoding method 430. The computer may reduce or eliminate degeneracy, for example, as part of the encoding method 410. Similarly, a computer can generate coding schemes that are non-degenerate by design, such as coding schemes designed using mathematical iteration. Computers can also be connected to network 405. The network may be a local area network and/or the Internet. A network can be used to perform the method of the present invention on multiple computers in different locations. For example, one computer can perform encoding, another computer can perform measurement, and yet another computer can perform decoding. A computer can perform any or all of these methods. Networks may be used to transmit information obtained using the methods described in this disclosure throughout the world, such as across national borders.

[0207]

2045 In one example, the software is embedded in the instrument that performs the measurements, such as a thermal cycler.

2047 In another example, the software is installed on a computer connected to the instrument performing the measurements, such as a computer connected to a thermal cycler. In another instance, the software is "in the cloud," that is, on another computer that can communicate with it over a network.

[0208]

2053 Each element described above can be connected to a controller, which communicates with each element and

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coordinates their operation.

2055 For example, the controller can initiate encoding of a series of analytes into color and intensity values. 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 can then automatically order probes corresponding to that code from the probe's manufacturer. Alternatively, the controller can operate an oligonucleotide synthesizer that can synthesize corresponding probes. After analyzing the sample on the instrument (which can also be automated), the controller can process the results of the analysis using the decoding matrix. The controller can then provide these results to the user.

[0209]

2065 VIII.

2066 Serve

[0210]

2070 The methods provided in this article can also be executed as services.

2071 For example, a service provider may obtain the identities of multiple analytes that a customer wishes to analyze. The service provider can then encode each analyte to be detected by any of the methods described herein and provide the customer with the appropriate reagents for the assay. The client can also perform measurements and provide the results to the service provider for decoding. The service provider can then provide the decoded results to the client. Customers can 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 over a network). Exemplary customers include clinical laboratories, physicians, food and consumer product manufacturers, industrial manufacturers (eg, oil companies), and the like. The customer or party may be any suitable customer or party who has a need or desire to use the methods, systems, compositions and kits of the invention.

[0211]

2084 Example

[0212]

2088 Example 1: General Materials and Methods

[0213]

2092 1.

2093 General reagents

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[0214]

2097 TE buffer, pH7 (LIFE TECHNOLOGIES, Carlsbad, CA); RNase-free ultrapure water (LIFE TECHNOLOGIES, Carlsbad, CA); Taq 5x master mix (FISHER SCIENTIFIC COMPANY, Tustin, CA).

[0215]

2102 2.

2103 DNA sequences, primers and probes

[0216]

2107 Five nucleic acids from clinically relevant organisms were selected for illustrative studies: (1) human immunodeficiency virus 1 (HIV-1); (2) Plasmodium falciparum (malaria); (3) herpes simplex virus 2 (HSV-2); (4) Mycobacterium tuberculosis (TB); and (5) Dengue virus type 3 (dengue fever).

[0217]

2113 Two diagnostically relevant regions from the HIV-1 genome, p17 and polyprotease, were selected from the Los Alamos National Laboratory HIV-1 reference sequence.

2115 Diagnostic sequences from the P. falciparum ChR7 gene were obtained from the UCSC P. falciparum genome browser.

2117 Herpes simplex virus 2 sequences were synthesized from sequences obtained from the European Molecular Biology Library.

2119 Similarly, diagnostic sequences for the rpoB gene in Mycobacterium tuberculosis were synthesized from sequences obtained from the European Molecular Biology Library.

2121 PCR diagnostic sequences for dengue virus type 3 were obtained from the National Institute of Health's Health Gene Sequence Database.

[0218]

2126 Oligonucleotides are synthesized from INTEGRATED DNA TECHNOLOGIES (IDT).

2127 Probe and primer pairs for each analyte were selected using the OLIGO analyzer from IDT.

2128 Sensing 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 the sequence information, target sequences, primers, and probes for each of the six analytes.

[0219]

2134 Table 9.

2135 Sequence information of HIV-1 polyprotease.

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[0220]

2139

[0221]

2144

[0222]

2149 Table 10.

2150 Sequence information of HIV p17.

[0223]

2154

[0224]

2159 Table 11.

2160 Sequence information of Plasmodium ChR 7.

[0225]

2164

[0226]

2169 Table 12.

2170 Sequence information for herpes simplex virus 2.

[0227]

2174

[0228]

2179 Table 13.

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2180 Sequence information of Mycobacterium tuberculosis rpoB.

[0229]

2184

[0230]

2189 Table 14.

2190 Sequence information of dengue virus type 3.

[0231]

2194

[0232]

2199

[0233]

2204 3.

2205 polymerase chain reaction

[0234]

2209 All PCR reactions were performed on a ROCHE 480LIGHTCYCLER instrument.

2210 Perform 45 cycles with a 60-second hot start at 95° C.

2211 The cycle conditions for denaturation, annealing and extension were 45 seconds, 50 seconds and 60 seconds at 95° C, 65° C and 70° C respectively.

2213 Each experiment was run in five replicates with a reaction volume of 15 μL.

2214 After each annealing step, fluorescence measurements were obtained in the following channels: 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).

2216 Fluorescence measurements are also available after a hot start and after 45 thermal cycles.

2217 The change in fluorescence intensity between these two measurements (after a hot start, and at the end of 45 cycles) determines the end-point fluorescence signal.

[0235]

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2222 Example 2: Simultaneous detection of polynucleotide markers from pathogens using more than one color coding method per analyte

[0236]

2227 This example describes the simultaneous detection of up to four polynucleotides selected from HIV (2 polynucleotides), HSV-2, Mycobacteria, Plasmodium, and Dengue virus.

2229 Fourteen PCR reactions were assembled using the reagents described in Table 15.

[0237]

2233 Table 15. Reagents for 14 PCR reactions.

[0238]

2237

[0239]

2242

[0240]

2247

[0241]

2252

[0242]

2257

[0243]

2262 Referring to Table 15, reactions 1, 3, 5, 7, and 9 (containing analytes for HIV polyprotease, Plasmodium, HSV, mycobacteria, and dengue, respectively) were generated for each polynucleotide in the absence of

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other analytes. Analytes alone provide a positive control for baseline fluorescence intensity.

2265 As described more fully below, the change in fluorescence intensity in each color is used to provide the expected cumulative intensity level for each color in the color spectrum (Figure 3).

2267 Reactions 2, 4, 6, 8, and 10 are negative controls, respectively, used to determine the extent of nonspecific amplification.

2269 In these reactions, the primer pair provided is specific to the analyte provided.

[0244]

2273 Experiments 11, 12, 13 and 14 are multiplex detection experiments.

2274 The encoding is performed as shown in Table 16.

2275 Within each color, the fluorescence intensity measured for each assay is depicted as a black circle in the chromatogram of Figure 3.

[0245]

2280 Table 16.

2281 Coding scheme for pathogen detection.

[0247]

2285 To construct the chromatogram, in each color, the signals of the positive controls (reactions 1, 3, 5, 7 and 9) were used to assemble every possible combination of sequences present.

2287 The accumulated signal is plotted in a graph called a "chromatogram" (Figure 3).

2288 The accumulated signal corresponding to all possible combinations of the same rank in the same color is organized into its own "band".

2290 Doing this for all four colors produces level and band structures against which the experimental results for each combination can be evaluated (Reactions 11, 12, 13 and 14).

2292 Figure 3 shows three replicates of a chromatogram constructed using a positive control sample with experimental results 4112, 3121 and 3102 superimposed on the chromatogram.

2294 Experimental results are indicated by black dots.

[0248]

2298 The intensity value of each color in reactions 11-14 was measured in arbitrary fluorescence units (AFU), plotted in a chromatogram, and then assigned to bands based on two criteria.

2300 First, if the experimental result is within a band, then the result is assigned to the multiplexed band (eg, a Cy3 value of 1 in the leftmost chromatogram of Figure 3).

2302 Second, if the experimental results are between two bands, a two-step analysis is used.

2303 If the experimental result is between two reasonable results, then the experimental result is assigned to the closest band (e.g., a value of 2 for Cy5 in the rightmost chromatogram of Figure 3).

2305 If the experimental result is between a reasonable result and an unreasonable result, the result is assigned to

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the reasonable result band (e.g., FAM value 3 in the middle chromatogram of Figure 3).

2307 Figure 8 schematically shows the reasonable results for 4112 (top panel), indicating the presence of HIV polyprotease, Plasmodium, herpes simplex, and mycobacteria; and the unreasonable results for 4110 (bottom panel), It cannot be decoded and indicates a failure in the measurement.

[0249]

2313 Using these criteria, the output of Experiment 12 is assigned 4112, which is the correct answer.

2314 The output of Experiment 14 could be specified as 2121 or 3121, but 2121 is unreasonable, so the result must be 3121, which is the correct answer. The output of Experiment 13 could be assigned as 3102 or 3101, both of which are reasonable, but in the Cy5 channel the result is definitely closer to 2 than 1, so the correct answer, 3102, is assigned as the result. These results provide experimental validation of the method presented in this article.

[0250]

2322 The results showed a tendency for the combined signal to be slightly lower than what would be expected from the simple sum of the signals from the positive controls.

2324 This may be because the combination of signals is not perfectly linear. This may be the result of additional effects of the chromatographic construction that are currently unexplained. These effects may be related to the absolute concentration of the quencher. The higher the absolute concentration of the quencher, the greater the percentage of the reaction volume it quenches, so that the same percentage of fluorophore released does not contribute as much to the accumulated signal as expected. One solution to this problem is to use a low concentration of probe such that the percent loss in signal is negligible, regardless of how much quencher is released.

[0251]

2334 Figure 3 shows that each rank contains multiple combinations with relatively broad bands.

2335 The width of the band is ultimately the difference between the highest and lowest accumulated signal of the positive control sample. If all positive controls produced exactly the same fluorescent signal in a particular color, the width of each band would be zero. Because the substituted signals are different, the resulting bands are relatively broad. However, there is a simple means to tighten the straps. As done in the experiment above, instead of loading all probes at the same concentration, the concentration of each probe can be adjusted so that each resulting positive control signal is the same as the others in the same color. This method significantly tightens the bands, making it easier to determine the signal multiplexing of samples containing analytes.

[0252]

2346 Example 3: Simultaneous detection of polynucleotide markers from pathogens using one color and different intensity encoding methods per analyte

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[0253]

2351 This example describes the simultaneous detection of up to three polynucleotides selected from dengue virus, HIV p17 and HIV polyprotease.

2353 The coding scheme is as depicted in Table 11.

2354 As shown in Table 11, each analyte is coded as a single value of fluorescence intensity in a single color. In this case, the encoding scheme is non-degenerate by design, as described elsewhere in this disclosure. This is accomplished by assigning an intensity value to each analyte equal to the cumulative intensity value of the previous analyte plus one. Of course, as described elsewhere in this specification, the initial assigned value may be greater than 1, for example, to better distinguish noise. Similarly, the value assigned to each analyte can be greater than the cumulative intensity value of the previous analyte plus one, for example, to increase the spacing between intensities and make intensity assignment simpler.

[0254]

2364 Table 17.

2365 Coding scheme for detecting dengue virus, HIV polyprotease and HIV p17.

[0256]

2369 The same reagents (eg, templates, primers, and probes) used in Example 2 were used here.

2370 The fluorescence signal produced by each probe in the positive control endpoint PCR reaction was measured and used to calculate the concentration of probes that would produce signal intensities of 1x, 2x, and 4x.

2372 The experiment is then set up to detect seven non-empty combinations of occurrences of the analyte. Tables 18-24 provide the analyte PCR reaction components for each of these mixtures.

[0257]

2377 Table 18.

2378 Binary FAM experiment 1 cocktail (Cocktail): HIV polyprotease, HIV p17, and dengue virus.

[0259]

2382 Table 19.

2383 Binary FAM Experiment 2 Mix: Dengue Virus and HIV p17.

[0261]

2387 Table 20.

2388 Binary FAM Experiment 3 Mix: Dengue Virus and HIV Polyprotease.

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[0263]

2392 Table 21.

2393 Binary FAM Experiment 4 Mix: HIV Polyprotease and HIV p17.

[0265]

2397 Table 22.

2398 Binary FAM Experiment 5 Mix: Dengue Virus.

[0268]

2402 Table 23.

2403 Binary FAM Experiment 6 Mix: HIV p17.

[0270]

2407 Table 24.

2408 Binary FAM Experiment 7 Mix: HIV Polyprotease.

[0273]

2412 The total fluorescence signal for each experiment was plotted on a chromatogram constructed as described above (Figure 7).

2414 The width of the band surrounding each positive control measurement is 1x the uncertainty in the propagation of the measurement.

2416 The uncertainty is equal to the standard deviation of the fluorescence signal over the last five PCR cycles of saturation.

[0274]

2421 Figure 7 shows the results of this experiment.

2422 The three results on the far right show the measured intensities of Dengue virus alone (DEN), HIV p17 alone (P17), and HIV polyprotease (TPP).

2424 The assigned intensities for each of these analytes are shown in Table 11, and Figure 7 confirms that the presence of the three analytes alone produced the expected intensity results (see DEN, P17, and TPP).

2426 At the end of the assay, substantially all of the probe has been hydrolyzed, releasing substantially all of the fluorophore emitting signal.

2428 A fixed number of fluorophores produces a fixed and predictable amount of signal.

2429 Therefore, the presence of target should generally produce a fixed amount of signal, determined by the amount of probe added to the reaction volume.

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[0275]

2434 As expected, the intensity value for a combination of analytes is equal to the sum of the assigned intensities.

2435 Because this coding scheme is designed to be non-degenerate, each result can be unambiguously decoded into the presence or absence of a specific analyte.

2437 For example, starting on the left side of Figure 7, the result for 7 indicates that all three analytes are present.

2438 The results for 3 indicate that dengue virus and HIV p17 analytes are present, but HIV polyprotease is not present.

2440 The results of 5 indicate that dengue virus and HIV polyprotease are present, but HIV p17 is not present.

2441 Finally, the results of 6 indicate that HIV p17 and HIV polyprotease are present but not dengue virus. This encoding scheme can be expanded to encode additional analytes as described elsewhere in this disclosure.

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Espacenet

CN104271767A Signal encoding and decoding in multiplexed biochemical assays

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Signal encoding and decoding in multiplexed biochemical assays

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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(54)发明名称

多路生化测定中信号的编码和解码

(57)摘要

本公开提供了用于样品中的多个分析物的多路检测的方法、系统、组合物、和试剂盒。在一些实例中，本公开提供了方法、系统、组合物、和试剂盒，其中，可在单个样品体积中通过获得信号的至少一个可计量的分量的累积测量或多个累积测量来检测多个分析物。在一些情况下，信号的额外分量、或额外的信号（或其分量）也被定量。每个信号或信号的每个分量可被用于构建编码方案，然后所述编码方案可用于确定任何分析物的存在或不存在。

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1. 一种测定，所述测定能够不进行固定化、分离、质谱法、或解链曲线分析而明确地检测单一样品体积中的、以存在或不存在的任意组合形式的至少 7 个分析物中的每个分析物的存在或不存在。

2. 根据权利要求 1 所述的测定，其中，每个所述分析物被编码为信号的一个分量的值。

3. 根据权利要求 2 所述的方法，其中，所述方法能够明确地检测以存在或不存在的任意组合形式的 M 个分析物的存在或不存在，其中 $M = \log_2(F+1)$，且 F 是当所有所述分析物存在时的所述信号的所述一个分量的最大累积值。

4. 根据权利要求 1 所述的测定，其中，每个所述分析物被编码为所述信号的第一分量中的至少一个第一值，及所述信号的第二分量中的至少一个第二值。

5. 根据权利要求 4 所述的测定，其中，每个所述分析物被编码为在所述信号的第二分量中的多个第二值中的每个第二值的所述信号的第一分量中的第一值。

6. 根据权利要求 4 所述的方法，其中，所述方法能够明确地检测以存在或不存在的任意组合形式的 M 个分析物的存在或不存在，其中 $M = C*\log_2(F+1)$，其中 C 是用于编码所述分析物的所述第二值的数目，且 F 是当所有所述分析物存在时，关于任意第二值的所述信号的所述第一分量的最大累积值。

7. 根据权利要求 4 所述的方法，其中，所述方法能够明确地检测以存在或不存在的任意组合形式的 M 个分析物的存在或不存在，其中 $M = (P*T)+1$，其中 P 是编码方案中每层代码的数目，且 T 是层的数目。

8. 根据权利要求 7 所述的方法，其中，所述层的数目 $T = \log4(F+1)$，且 F 是当所有所述分析物存在时，关于任意第二值的所述信号的所述第一分量的最大累积值。

9. 根据权利要求 4 所述的方法，其中，所述第一值是强度或强度的范围。

10. 根据权利要求 9 所述的方法，其中，所述编码方案包括至少三个强度或强度的范围。

11. 根据权利要求 4 所述的方法，其中，所述第二值是波长或波长的范围。

12. 根据权利要求 11 所述的方法，其中，所述编码方案包括至少五个波长或波长的范围。

13. 根据权利要求 1 所述的方法，其中，所述信号是电磁信号。

14. 根据权利要求 13 所述的方法，其中，所述电磁信号是荧光发射信号。

15. 根据权利要求 14 所述的方法，其中，测量了位于至少四个波长或波长的范围处的所述荧光发射信号的强度。

16. 根据权利要求 1 所述的方法，其中，所述检测利用使用之前被冻干的试剂执行。

17. 根据权利要求 1 所述的方法，其中，所述检测使用包括杂交探针的试剂执行。

18. 根据权利要求 17 所述的方法，其中，所述杂交探针的数目大于所述分析物的数目。

19. 根据权利要求 17 所述的方法，其中，所述杂交探针包括对不同分析物特异的一个或更多个杂交探针，且包括相同的荧光团或荧光团的组合。

20. 根据权利要求 17 所述的方法，其中，所述样品与至少 18 个所述杂交探针接触。

21. 根据权利要求 17 所述的方法，其中，所述试剂还包括能够扩增与至少三个所述杂交探针互补的区域的至少一对引物。

22. 根据权利要求 21 所述的方法，其中，所述信号是在聚合酶链式反应中产生的。

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23. 根据权利要求 22 所述的方法，其中，所述聚合酶链式反应选自以下所构成的组：终点聚合酶链式反应、实时聚合酶链式反应、及数字聚合酶链式反应。

24. 根据权利要求 1 所述的方法，其中，所述累积测量针对溶液执行。

25. 根据权利要求 1 的所述方法，其中，至少一种所述分析物是通过至少一个额外的值编码的，其中所述至少一个额外的值选自以下所构成的组：来自所述信号的至少一个额外的分量的值、来自额外的信号的至少一个分量的值、及其组合。

26. 根据权利要求 25 所述的方法，其中，所述至少一个额外的值是选自以下所构成的组：荧光发射强度、荧光发射波长、Förster 共振能量转移 FRET 发射强度、FRET 发射波长、电化学信号、化学发光波长、化学发光强度、荧光漂白率、化学发光漂白率、及其组合。

27. 根据权利要求 1 所述的方法，还包括构建色谱。

28. 根据权利要求 27 所述的方法，其中，通过绘制阳性对照样品的所述第一值和所述第二值的所有可能的组合来构建所述色谱。

29. 根据权利要求 1 所述的方法，其中，至少一个所述分析物包括多核苷酸。

30. 根据权利要求 29 所述的方法，其中，所述多核苷酸来自选自以下所构成的组的来源：动物、植物、细菌、真菌、寄生物和病毒。

31. 根据权利要求 23 所述的方法，其中，所述多核苷酸来自选自以下所构成的组的来源：人类免疫缺陷病毒、单纯性疱疹病毒、人类乳头瘤病毒、疟原虫、分枝杆菌、登革热病毒、肝炎病毒和流感病毒。

32. 根据权利要求 31 所述的方法，其中，所述多核苷酸选自以下所构成的组：人类免疫缺陷病毒多蛋白酶、人类免疫缺陷病毒 p17、人类乳头瘤病毒 E6、及人类乳头瘤病毒 E7。

33. 根据权利要求 1 所述的方法，其中，所述样品选自以下所构成的组：临床样品、食品样品、环境样品、药物样品、及来自消费产品的样品。

34. 根据权利要求 1 所述的方法，还包括通过计算机网络传输关于所述分析物存在或不存在的信息。

35. 根据权利要求 1 所述的方法，还包括向医生提供关于所述分析物存在或不存在的信息。

36. 根据权利要求 35 所述的方法，还包括基于所述信息做出临床决定。

37. 根据权利要求 1 所述的方法，其中，使用计算机可读介质上的指令执行所述方法的至少一个步骤。

38. 根据权利要求 37 所述的方法，其中，所述指令位于远程服务器上。

39. 根据权利要求 37 所述的方法，其中，所述指令位于热循环仪上。

40. 根据权利要求 37 所述的方法，其中，所述指令位于与热循环仪通信的计算机上。

41. 根据权利要求 1 所述的方法，其中，至少一个所述分析物是阳性对照分析物。

42. 根据权利要求 1 所述的方法，其中，所述编码方案是非简并的。

43. 根据权利要求 42 所述的方法，其中，所述编码方案被设计为非简并的。

44. 根据权利要求 42 所述的方法，其中，通过枚举可以从所述编码方案获得的每个合理的结果、鉴定是简并的每个合理的结果、并从所述编码方案消除至少一个潜在的分析物代码以消除简并性，所述编码方案被做成非简并的。

45. 根据权利要求 1 所述的方法，其中，所述测定是终点测定。

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46. 根据权利要求 1 所述的方法，其中，所述测定在仪器的检测极限设定的循环的阈值数目结束。

47. 根据权利要求 1 所述的方法，其中，所述测定是液相测定。

48. 根据权利要求 1 所述的方法，其中，所述测定是定量的。

49. 一种检测多个分析物中的每个分析物存在或不存在的方法，所述方法包括：

a. 将每个所述分析物编码为信号的第一值，由此产生编码方案，其中每个所述分析物在所述编码方案中由所述第一值表示，其中所述编码是以消除简并性的方式进行的；

b. 提供包括或可能包括至少一个所述分析物的样品；

c. 将所述样品与如在所述编码方案中表示的在每个所述分析物存在时产生所述第一值的分析物特异性试剂接触；

d. 累积地测量所述样品中所述信号的所述第一值，从而提供累积测量结果；及

e. 基于所述累积测量结果和所述编码方案，确定每个所述分析物是存在还是不存在。

50. 根据权利要求 49 所述的方法，其中，所述方法能够明确地检测以存在或不存在的任意组合形式的 M 个分析物的存在或不存在，其中 $M = \log_2(F+1)$，且 F 是当所有所述分析物存在时的所述第一值的最大累积值。

51. 根据权利要求 49 所述的方法，其中，所述第一值是强度或强度的范围。

52. 根据权利要求 49 所述的方法，其中，所述第一值具有在所述编码方案中的最小值，所述最小值选自以下所构成的组：至少 1、至少 2、至少 4、至少 8、至少 16、至少 32 和至少 64。

53. 根据权利要求 49 所述的方法，其中，所述第一值通过等于所述先前第一值的累积最大值加一的量在所述编码方案中增加。

54. 根据权利要求 49 所述的方法，其中，所述第一值通过大于所述先前第一值的累积最大值加一的量在所述编码方案中增加。

55. 一种检测多个分析物中的每个分析物存在或不存在的方法，所述方法包括：

a. 以至少一个第一值和至少一个第二值编码每个所述分析物，其中，所述第一值是来自信号的第一分量的值且所述第二值是来自所述信号的第二分量的值，从而产生编码方案，其中每个所述分析物在所述编码方案中通过所述至少一个第一值和所述至少一个第二值表示，其中所述编码以减少或消除简并性的方式进行；

b. 提供包括或可能包括至少一个所述分析物的样品；

c. 将所述样品与如在所述编码方案中表示的在每个所述分析物存在时产生所述至少一个第一值和至少一个所述第二值的分析物特异性试剂接触；

d. 累积地测量所述样品中的所述第一值和所述第二值，从而提供累积测量结果；及

e. 基于所述累积测量结果和所述编码方案，确定每个所述分析物是存在还是不存在，

其中，当简并性被消除时，在每个所述试剂仅产生一个第二值时，所述方法能够明确地检测单一体积中的、以存在或不存在的任意组合形式的至少 6 个分析物中的每个分析物的存在或不存在。

56. 根据权利要求 55 所述的方法，其中，每个所述分析物被编码为在所述信号的第二分量中的多个第二值中的每个第二值的所述信号的第一分量中的第一值。

57. 根据权利要求 55 所述的方法，其中，当简并性被消除时，所述方法能够使用 4 个第二值明确地检测单一体积中的至少 7 个分析物中的每个分析物的存在或不存在。

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58. 根据权利要求 55 所述的方法，其中，当简并性被消除时，所述编码方案包括 T 个非简并层，其中 $T = \log_4(F+1)$，且 F 是当所有所述分析物存在时，关于任意第二值的所述信号的所述第一分量的最大累积值。

59. 根据权利要求 56 所述的方法，其中，当简并性被消除时，所述方法能够明确地检测以存在或不存在的任意组合形式的 M 种分析物的存在或不存在，其中 $M = (P*T)+1$，且 P 是每层代码的数目。

60. 根据权利要求 55 所述的方法，其中，当简并性被消除时，所述方法能够明确地检测以存在或不存在的任意组合形式的 M 个分析物的存在或不存在，其中 $M = C*\log_2(F+1)$，C 是所述编码方案中第二值的数目，且 F 是当所有所述分析物存在时，关于任意第二值的所述信号的所述第一分量的最大累积值。

61. 一种非简并编码方案，所述非简并编码方案能够不执行固定化、分离、或解链曲线分析而明确地编码单一样品体积中的、以存在或不存在的任意组合形式的至少 7 个分析物中的每个分析物的存在或不存在。

62. 根据权利要求 61 所述的非简并编码方案，其中，所述编码方案由一种方法产生，所述方法包括：

a. 产生用于每个潜在分析物的代码，其中，每个潜在的分析物通过信号的至少一个分量的至少一个值编码；

b. 枚举每个分析物的存在或不存在的所有可能组合的每个合理的累积结果；

c. 鉴定每个合理的简并的结果；及

d. 消除至少一个代码，以消除简并性。

63. 根据权利要求 62 所述的方法，还包括通过增加额外的代码来扩展所述编码方案，所述额外的编码比所述信号的所述至少一个分量中的所有先前的代码的总和大至少一个单位。

64. 根据权利要求 61 所述的方法，其中，所述编码方案通过由结构保证非简并性的数学迭代的方法产生。

65. 一种用于检测多个分析物中的每个分析物存在或不存在的系统，所述系统包括：

a. 将每个所述分析物编码为信号的第一值，由此产生编码方案，其中每个所述分析物在所述的编码方案中由所述第一值表示，其中所述编码以消除简并性的方式进行；

b. 提供包括或可能包括至少一个所述分析物的样品；

c. 将所述样品与如所述编码方案中表示的在每个所述分析物存在时产生所述第一值的分析物特异性试剂接触；

d. 累积地测量所述样品中所述信号的所述第一值，从而提供累积测量结果；及

e. 基于所述累积测量结果和所述编码方案，确定每个所述分析物是存在还是不存在。

66. 一种用于检测多个分析物中的每个分析物存在或不存在的系统，所述系统包括：

a. 以至少一个第一值和至少一个第二值编码每个所述分析物，其中所述第一值是来自信号的第一分量的值且所述第二值是来自所述信号的第二分量的值，从而产生编码方案，其中每个所述分析物在所述编码方案中通过所述至少一个第一值和所述至少一个第二值表示，其中所述编码以减少或消除简并性的方式进行；

b. 提供包括或可能包括至少一个所述分析物的样品；

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c. 将所述样品与如所述编码方案中表示的在每个所述分析物存在时产生所述至少一个第一值和所述至少一个第二值的分析物特异性试剂接触；

d. 累积地测量所述样品中的所述第一值和所述第二值，从而提供累积测量结果；及

e. 基于所述累积测量结果和所述编码方案，确定每个所述分析物是存在还是不存在，

其中，当简并性被消除时，在每个所述试剂仅产生一个第二值时，所述系统能够明确地检测单一体积中的、以存在或不存在的任意组合形式的至少 6 个分析物中的每个分析物的存在或不存在。

67. 一种检测多个分析物中的每个分析物存在或不存在的方法，所述方法包括：

a. 从团体获得每个所述分析物的标识；

b. 将每个所述分析物编码为信号的第一值，由此产生编码方案，其中每个所述分析物在所述编码方案中由所述第一值表示，其中所述编码以消除简并性的方式进行；

c. 向所述团体提供如所述编码方案中表示的、当每个所述分析物存在时产生所述第一值的分析物特异性试剂，所述团体：

i. 将包括或可能包括至少一个所述分析物的样品与所述试剂接触；及

ii. 累积地测量所述样品中的所述第一值，从而提供累积测量结果；及

d. 从所述团体获得所述累积测量结果；

e. 基于所述累积测量结果和所述编码方案，确定每个所述分析物是存在还是不存在；及

f. 将关于每个所述分析物存在或不存在的信息提供给所述团体。

68. 一种检测多个分析物中的每个分析物存在或不存在的方法，所述方法包括：

g. 从团体获得每个所述分析物的标识；

h. 将每个所述分析物编码为至少一个第一值和至少一个第二值，其中，所述第一值是来自信号的第一分量的值，且所述第二值是来自所述信号的第二分量的值，由此产生编码方案，其中每个所述分析物在所述编码方案中由所述至少一个第一值和所述至少一个第二值表示，其中所述编码以减少或者消除简并性的方式进行；

i. 向所述团体提供如所述编码方案中表示的在每个所述分析物存在时产生所述至少一个第一值和所述至少一个第二值的分析物特异性试剂，所述团体：

i. 将包括或可能包括至少一个所述分析物的样品与所述试剂接触；及

ii. 累积地测量所述样品中的所述第一值和所述第二值，从而提供累积测量结果；及

j. 从所述团体获得所述累积测量结果；

k. 基于所述累积测量结果和所述编码方案，确定每个所述分析物是存在还是不存在；及

l. 将关于每个所述分析物存在或不存在的信息提供给所述团体。

69. 一种用于检测多个分析物中的每个分析物的存在或不存在的组合物，所述组合物包括分析物特异性试剂，每个试剂产生包括第一值的信号，其中所述试剂能够不执行固定化、分离、或解链曲线分析而明确地检测单一样品体积中的、以存在或不存在的任意组合形式的至少 7 个分析物中的每个分析物的存在或不存在。

70. 一种用于检测多个分析物中的每个分析物的存在或不存在的组合物，所述组合物包括分析物特异性试剂，每个试剂产生包括至少一个第一值和至少一个第二值的信号，所

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述至少一个第一值是来自所述信号的第一分量的值且所述至少一个第二值是来自所述信号的第二分量的值，其中，当每个所述试剂仅产生一个第二值时，所述试剂能够检测单一体积中的、以存在或不存在的任意组合形式的至少 6 个分析物中的每个分析物的存在或不存在。

71. 一种用于检测多个分析物中的每个分析物的存在或不存在的试剂盒，所述试剂盒包括分析物特异性试剂、包装、及说明书，每个试剂产生包括第一值的信号，其中，所述试剂盒能够不执行固定化、分离、或解链曲线分析而明确地检测单一样品体积中的、以存在或不存在的任意组合形式的至少 7 个分析物中的每个分析物的存在或不存在。

72. 一种用于检测多个分析物中的每个分析物的存在或不存在的试剂盒，所述试剂盒包括分析物特异性试剂、包装、及说明书，每个试剂产生包括至少一个第一值和至少一个第二值的信号，所述至少一个第一值是来自所述信号的第一分量的值且所述至少一个第二值是来自所述信号的第二分量的值，其中，当每个所述试剂仅产生一个第二值时，所述试剂盒能够检测单一体积中的、以存在或不存在的任意组合形式的至少 6 个分析物中的每个分析物的存在或不存在。

73. 一种用于检测多个分析物中的每个分析物存在或不存在的试剂盒，所述试剂盒包括盒体 (601)、布置在所述盒体中用于放置瓶的卡接槽口、包括用于分析物的检测的探针的瓶 (602)、包括用于扩增的引物的瓶 (603)、及包括用于扩增的试剂的瓶 (604)，其中，所述试剂盒能够不执行固定化、分离、或解链曲线分析而明确地检测单一样品体积中的、以存在或不存在的任意组合形式的至少 7 个分析物中的每个分析物的存在或不存在。

74. 一种用于检测多个分析物中的每个分析物存在或不存在的试剂盒，包括：盒体 (601)、布置在所述盒体中用于放置瓶的卡接槽口、包括用于分析物的检测的探针的瓶 (602)、包括用于扩增的引物的瓶 (603)、及包括用于扩增的试剂的瓶 (604)，每个探针产生包括至少一个第一值和至少一个第二值的信号，所述至少一个第一值是来自所述信号的第一分量的值且所述至少一个第二值是来自所述信号的第二分量的值，其中，当每个所述探针仅产生一个第二值时，所述试剂盒能够检测单一体积中的、以存在或不存在的任意组合形式的至少 6 个分析物中的每个分析物的存在或不存在。

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多路生化测定中信号的编码和解码

[0001] 交叉引用

[0002] 本申请要求 2012 年 2 月 3 日提交的美国临时申请 61/594,480, 及 2012 年 9 月 19 日提交的美国临时申请 61/703,093 的利益，其各自通过引用其全文并入本文。

[0003] 背景

[0004] 相比传统的单路反应，多路反应提供了显著的优点，包括在相同样品上执行并行的多个反应，使用相同的室执行更多个反应，以及以快速且有效的方式从样品中提取丰富信息的能力。然而，为了实现这些优点，多路测定通常需要复杂的报告机制，即光谱上可分辨的荧光或化学发光（例如，PCR、ELISA）、空间上可分辨的信号（例如，微阵列、凝胶电泳）、时间上可分辨的信号（例如，毛细管电泳）或其组合（例如，Sanger 测序）。对可以在单一的溶液中执行的多路反应是有需求的。

[0005] 发明概述

[0006] 本公开提供了用于分析物的多路检测的方法、组合物、系统和试剂盒。在一些情况下，本公开提供测定，所述测定能够不执行固定化 (immobilization)、分离、质谱法、或解链 (melting) 曲线分析而明确地检测单一样品体积中的、以存在或不存在的任意组合形式的至少 7 种分析物中的每种分析物的存在或不存在。在一些实例中，每种分析物被编码为信号的一个（或至少一个）分量的值。

[0007] 在一些实例中，本公开提供的测定能够明确地检测以存在或不存在的任意组合形式的、M 种分析物的存在或不存在，其中 $M = \log_2(F+1)$，且 F 是当所有分析物存在时的信号的一个分量（例如，强度）的最大累积值。

[0008] 在一些情况下，每种分析物被编码为信号的第一分量中的至少一个第一值（例如，至少一个强度或强度范围）和信号的第二分量中的至少一个第二值（例如，至少一个波长或波长范围）。在一些实例中，每种分析物被编码为在信号的第二分量中的多个第二值中的每个第二值的信号的第一分量中的第一值（例如，在多个波长或多个波长范围中的每个波长或每个波长范围的信号强度或信号强度范围）。

[0009] 在一些实例中，本文提供的测定能够明确地检测以存在或不存在的任意组合形式的、M 种分析物的存在或不存在，其中 $M = C*\log_2(F+1)$，其中 C 是用于编码分析物的第二值的数目，且 F 是在所有分析物存在时的、关于任意第二值的信号的第一分量的最大累积值。

[0010] 在一些情况下，本公开提供的测定能够明确地检测以存在或不存在的任意组合形式的、M 种分析物的存在或不存在，其中 $M = (P*T)+1$，其中 P 是编码方案中每层代码的数目，且 T 是层的数目。在一些情况下，层的数目 $T = \log_4(F+1)$，且 F 是在所有分析物存在时的、关于任意第二值的信号的第一分量的最大累积值。

[0011] 在一些实例中，第一值是强度或强度范围。在一些情况下，编码方案包括至少三个强度或强度范围。

[0012] 在一些情况下，第二值是波长或波长范围。在一些实例中，编码方案包括至少五个波长或波长范围。

[0013] 在一些实施例中，信号是电磁信号。在一些情况下，电磁信号是荧光发射信号。在

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一些实施例中，测量了在至少四个波长或波长范围处的荧光发射信号的强度。

[0014] 在一些情况下，本文提供的测定利用在使用之前被冻干的试剂执行。

[0015] 在一些情况下，利用包括杂交探针的试剂执行检测步骤。在一些实例中，杂交探针的数目大于分析物的数目。在一些情况下，杂交探针组包括专门用于不同分析物的一个或更多个杂交探针，且包括相同的荧光团或荧光团的组合。在一些实例中，样品与至少 18 个所述杂交探针接触。在一些实例中，利用包括至少一对引物的试剂执行检测步骤。在一些情况下，至少一对引物能够扩增与至少三个所述杂交探针互补的区域。

[0016] 在一些实例中，在聚合酶链式反应期间产生被测量的信号。在一些情况下，聚合酶链式反应选自以下所构成的组：终点 (end-point) 聚合酶链式反应、实时聚合酶链式反应、数字聚合酶链式反应、及其组合。

[0017] 在一些实例中，针对溶液执行至少一个累积测量。

[0018] 在一些实例中，至少一种分析物是通过至少一个额外的值（即，至少两个值一起）编码的，其中，至少一个额外的值选自以下所构成的组：来自信号的至少一个额外的分量的值，来自不同的信号的至少一个分量的值，及其组合。例如，至少一个额外的值可选自以下所构成的组：荧光发射强度、荧光发射波长、Förster 共振能量转移 (FRET) 发射强度、FRET 发射波长、电化学信号、化学发光波长、化学发光强度、荧光漂白率、化学发光漂白率、及其组合。

[0019] 在一些情况下，色谱被构建。通过绘制每个分析物的阳性对照 (positive control) 样品的第一值和第二值的所有可能的组合可构建色谱。

[0020] 在一些实例中，至少一个分析物包括多核苷酸。在一些情况下，多核苷酸来自选自以下所构成的组的来源：动物、植物、细菌、真菌、寄生物和病毒。在一些实例中，多核苷酸来自选自以下所构成的组的来源：人类免疫缺陷病毒、单纯性疱疹病毒、人类乳头瘤病毒、疟原虫、分枝杆菌、登革热病毒、肝炎病毒和流感病毒。在一些情况下，多核苷酸选自以下所构成的组：人类免疫缺陷病毒多蛋白酶 (polyprotease)、人类免疫缺陷病毒 p17、人类乳头瘤病毒 E6、及人类乳头瘤病毒 E7。

[0021] 在一些情况下，样品选自以下所构成的组：临床样品、食品样品、环境样品、药物样品、及来自消费产品的样品。

[0022] 在一些实例中，关于分析物存在或不存在的信息通过计算机网络传输。

[0023] 在一些情况下，向医生提供关于分析物存在或不存在的信息。在一些实例中，临床决定是基于此类信息做出的。

[0024] 在一些实例中，本文提供的方法的至少一个步骤是使用计算机可读介质上的指令执行的。在一些情况下，指令位于远程服务器上。在一些实例中，指令位于热循环仪上。在一些情况下，指令位于与热循环仪通信的计算机上。

[0025] 在一些情况下，至少一个分析物是阳性对照分析物。

[0026] 在一些实例中，编码方案是非简并的。在一些情况下，编码方案被设计为非简并的。在一些实例中，通过枚举可以从编码方案获得的每个合理的结果、鉴定是简并的每个合理的结果、并消除来自编码方案的至少一个潜在的分析物代码以消除简并性，编码方案被做成非简并的。

[0027] 在一些情况下，本文提供的测定是终点测定。在一些实例中，本公开提供的测定在

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通过仪器的检测极限设定的循环的阈值数结束。

[0028] 在一些实例中，本文提供的测定是液相测定。

[0029] 在一些情况下，本文提供的测定是定量的。

[0030] 在一些情况下，本公开提供了检测多个分析物中的每个分析物存在或不存在的方法，所述方法包括：(a) 将每个所述分析物编码为信号的第一值，由此产生编码方案，其中每个所述分析物在所述编码方案中由所述第一值表示，其中所述编码是以消除简并性的方式执行的；(b) 提供包括或可能包括至少一个所述分析物的样品；(c) 将所述样品与如所述编码方案中表示的在每个所述分析物存在时产生所述第一值的分析物特异性试剂接触；(d) 累积地测量所述样品中所述信号的所述第一值，从而提供累积测量结果；及 (e) 基于所述累积测量结果和所述编码方案，确定每个所述分析物是存在还是不存在。

[0031] 在一些实例中，在先前段落描述的方法能够明确地检测以存在或不存在的任意组合的 M 种分析物的存在或不存在，其中 $M = \log_2(F+1)$，且 F 是当所有分析物存在时的第一值的最大累积值。在一些情况下，第一值是强度或强度范围。在一些实例中，第一值具有在编码方案中的最小值，所述最小值选自以下所构成的组：至少 1、至少 2、至少 4、至少 8、至少 16、至少 32 和至少 64。在一些情况下，第一值通过等于所述先前第一值的累积最大值的加一的量在编码方案中增加。在其它情况下，第一值通过大于先前第一值的累积最大值加一的量在编码方案中增加。

[0032] 在一些情况下，本公开提供了检测多个分析物中的每个分析物存在或不存在的方法，所述方法包括：(a) 以至少一个第一值和至少一个第二值编码每个所述分析物，其中所述第一值是来自信号的第一分量的值且所述第二值是来自所述信号的第二分量的值，从而产生编码方案，其中每个所述分析物在所述编码方案中通过所述至少一个第一值和所述至少一个第二值表示，其中所述编码以减少或消除简并性的方式执行；(b) 提供包括或可能包括至少一种所述分析物的样品；(c) 将所述样品与所述编码方案中表示的在每个所述分析物存在时产生所述至少一个第一值和至少一个所述第二值的分析物特异性试剂接触；(d) 累积地测量所述样品中的所述第一值和所述第二值，从而提供累积测量结果；及 (e) 基于所述累积测量结果和所述编码方案，确定每个所述分析物是存在还是不存在，其中，当简并性被消除时，在每个所述试剂仅产生一个第二值时，所述方法能够明确地检测单一体积中的、以存在或不存在的任意组合形式的、至少 6 个分析物的每个分析物的存在或不存在。

[0033] 在一些情况下，在先前段落描述的方法中的每个分析物被编码为在信号的第二分量中的多个第二值中的每个第二值的信号的第一分量中的第一值。在一些情况下，当简并性被消除时，该方法使用 4 个第二值能够明确地检测单一体积中的至少 7 个分析物中的每个分析物的存在或不存在。在一些实例中，当简并性被消除时，编码方案包括 T 个非简并层，其中 $T = \log_4(F+1)$，且 F 是当所有分析物存在时，关于任意第二值的信号的第一分量的最大累积值。在一些情况下，当简并性被消除时，方法能够明确地检测以存在或不存在的任意组合形式的、M 种分析物的存在或不存在，其中 $M = (P*T)+1$，且 P 是每层代码的数目。在一些实例中，当简并性被消除时，方法能够明确地检测以存在或不存在的任意组合形式的、M 种分析物的存在或不存在，其中 $M = C*\log_2(F+1)$，C 是编码方案中第二值的数目，且 F 是当所有分析物存在时，关于任意第二值的信号的第一分量的最大累积值。

[0034] 本公开还提供了非简并编码方案，所述非简并编码方案能够不执行固定化、分离、

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质谱法、或解链曲线分析而明确地编码单一样品体积中的、以存在或不存在的任意组合的至少 7 个分析物中的每个分析物的存在或不存在。

[0035] 在一些实例中，本公开的非简并编码方案是通过包括以下步骤的方法产生的：(a) 产生用于每个潜在分析物的代码，其中每个潜在的分析物通过信号的至少一个分量的至少一个值编码；(b) 枚举对存在或不存在的每个分析物的所有可能组合的每个合理的累积结果；(c) 鉴定每个合理的简并的结果；及 (d) 消除至少一个代码，以消除简并性。在一些情况下，编码方案可通过增加额外的代码扩大，所述额外的编码比所述信号的至少一种分量中的所有先前的代码的总和大至少一个单位。在一些情况下，编码方案通过由结构保证非简并性的数学迭代的方法产生。

[0036] 在一些情况下，本公开提供了用于检测多个分析物中的每个分析物存在或不存在的系统，所述系统包括：(a) 编码每个所述分析物为信号的第一值，由此产生编码方案，其中每个所述分析物在所述的编码方案中由所述第一值表示，其中所述编码是以消除简并性的方式执行的；(b) 提供包括或可能包括至少一个所述分析物的样品；(c) 将所述样品与如在所述编码方案中表示的、当每个所述分析物存在时产生所述第一值的分析物特异性试剂接触；(d) 累积地在所述样品中测量所述信号的所述第一值，从而提供累积测量结果；及 (e) 基于所述累积测量结果和所述编码方案，确定每个所述分析物是存在还是不存在。

[0037] 在一些情况下，本公开提供了用于检测多个分析物中的每个分析物存在或不存在的系统，所述系统包括：(a) 以至少一个第一值和至少一个第二值编码每个所述分析物，其中所述第一值是来自信号的第一分量的值且所述第二值是来自所述信号的第二分量的值，从而产生编码方案，其中每个所述分析物在所述编码方案中通过所述至少一个第一值和所述至少一个第二值表示，其中所述编码以减少或消除简并性的方式执行；(b) 提供包括或可能包括至少一种所述分析物的样品；(c) 将所述样品与在所述编码方案中表示的、当每个所述分析物存在时产生所述至少一个第一值和所述至少一个第二值的分析物特异性试剂接触；(d) 累积地测量所述样品中的所述第一值和所述第二值，从而提供累积测量结果；及 (e) 基于所述累积测量结果和所述编码方案，确定每个所述分析物是存在还是不存在，其中，当简并性被消除时，在每个所述试剂仅产生一个第二值时，所述方法能够明确地检测在单一体积中的、以存在或不存在的任意组合形式的、至少 6 个分析物的每个分析物的存在或不存在。

[0038] 在一些情况下，本公开提供了用于第三团体的检测多个分析物中的每个分析物存在或不存在的方法，所述方法包括：(a) 从团体获得每个所述分析物的标识；(b) 将每个所述分析物编码为信号的第一值，由此产生编码方案，其中每个所述分析物在所述的编码方案中由所述第一值表示，其中所述编码以消除简并性的方式执行；(c) 向所述团体提供如在所述编码方案中表示的、当每个所述分析物存在时产生所述第一值的分析物特异性试剂，所述团体：(i) 将包括或可能包括至少一个所述分析物的样品与所述试剂接触；及 (ii) 累积地测量所述样品中的所述第一值，从而提供累积测量结果；及 (d) 从所述团体获得所述累积测量结果；(e) 基于所述累积测量结果和所述编码方案，确定每个所述分析物是存在还是不存在；及 (f) 将关于每个所述分析物存在或不存在的信息提供给所述团体。

[0039] 在一些情况下，本公开提供了用于第三团体的检测多个分析物中的每个分析物存在或不存在的方法，所述方法包括：(a) 从团体获得每个所述分析物的标识；(b) 将每个所

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述分析物编码为至少一个第一值和至少一个第二值，其中，所述第一值是来自信号的第一分量的值，且所述第二值是来自所述信号的第二分量的值，由此产生编码方案，其中每个所述分析物在所述的编码方案中由所述至少一个第一值和所述至少一个第二值表示，其中所述编码以减少或者消除简并性的方式执行；(c) 向所述团体提供如所述编码方案中表示的在每个所述分析物存在时产生所述至少一个第一值和所述至少一个第二值的分析物特异性试剂，所述团体：(i) 将包括或可能包括至少一个所述分析物的样品与所述试剂接触；及 (ii) 累积地测量所述样品中所述第一值及所述第二值，从而提供累积测量结果；及 (d) 从所述团体获得所述累积测量结果；(e) 基于所述累积测量结果和所述编码方案，确定每个所述分析物是存在还是不存在；及 (f) 将关于每个所述分析物存在或不存在的信息提供给所述团体。

[0040] 在一些情况下，本公开提供了用于检测多个分析物中的每个分析物的存在或不存在的组合物，所述组合物包括分析物特异性试剂，每个试剂产生包括第一值的信号，其中所述试剂能够在不执行固定化、分离、质谱法、或解链曲线分析的情况下，明确地检测单一样品体积中的、以存在或不存在的任意组合形式的、至少 7 个分析物的每个分析物的存在或不存在。

[0041] 在一些情况下，本公开提供了用于检测多个分析物中的每个分析物的存在或不存在的组合物，所述组合物包括分析物特异性试剂，每个试剂产生包括至少一个第一值和至少一个第二值的信号，所述至少一个第一值是来自所述信号的第一分量的值且所述至少一个第二值是来自所述信号的第二分量的值，其中，当每个所述试剂仅产生一个第二值时，所述试剂能够检测在单一体积中的、以存在或不存在的任意组合形式的、至少 6 个分析物的每个分析物的存在或不存在。

[0042] 在一些情况下，本公开提供了用于检测多个分析物中的每个分析物的存在或不存在的试剂盒，所述试剂盒包括分析物特异性试剂、包装、及说明书，每个试剂产生包括第一值的信号，其中所述试剂盒能够在不执行固定化、分离、质谱法、或解链曲线分析的情况下，明确地检测单一样品体积中的、以存在或不存在的任意组合形式的、至少 7 个分析物的每个分析物的存在或不存在。

[0043] 在一些实例中，本公开提供了用于检测多个分析物中的每个分析物的存在或不存在的试剂盒，所述试剂盒包括分析物特异性试剂、包装、及说明书，每个试剂产生包括至少一个第一值和至少一个第二值的信号，所述至少一个第一值是来自所述信号的第一分量的值且所述至少一个第二值是来自所述信号的第二分量的值，其中，当每个所述试剂仅产生一个第二值时，所述试剂盒能够检测在单一体积中的、以存在或不存在的任意组合形式的、至少 6 个分析物中的每个分析物的存在或不存在。

[0044] 在一些情况下，本公开提供了用于检测多个分析物中的每个分析物存在或不存在的试剂盒，所述试剂盒包括盒体 (601)、布置在盒体中用于放置瓶的卡接槽口、包括用于分析物的检测的探针的瓶 (602)、包括用于扩增的引物的瓶 (603)、及包括用于扩增的试剂的瓶 (604)、其中，所述试剂盒能够在不执行固定化、分离、质谱法、或解链曲线分析的情况下，明确地检测单一样品体积中的、以存在或不存在的任意组合形式的至少 7 个分析物中的每个分析物的存在或不存在。

[0045] 在一些实例中，本公开提供了用于检测多个分析物中的每个分析物存在或不存在

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的试剂盒，包括：盒体 (601)、布置在盒体中用于放置瓶的卡接槽口、包括用于分析物的检测的探针的瓶 (602)、包括用于扩增的引物的瓶 (603)、及包括用于扩增的试剂的瓶 (604)，每个探针产生包括至少一个第一值和至少一个第二值的信号，所述至少一个第一值是来自所述信号的第一分量的值且所述至少一个第二值是来自所述信号的第二分量的值，其中，当每个所述探针仅产生一个第二值时，所述试剂盒能够检测在单一体积中的、以存在或不存在的任意组合形式的至少 6 个分析物中的每个分析物的存在或不存在。

[0046] 引用并入

[0047] 在本说明书中提到的所有的出版物和专利申请在此通过引用以相同程度并入，如同每个单独的出版物或专利申请被具体地和单独地表明通过引用并入。

[0048] 附图简述

[0049] 本发明的新颖特征在所附权利要求书中特别陈述。将通过参考以下的详细描述的说明性实施方案的陈述获得对本发明的特征和优点更好地理解，其中本发明的原理被利用，且其中的附图是：

[0050] 图 1 显示了以四个颜色检测四个分析物的传统的编码方法，及以四个颜色能够检测 16 个或更多个序列的本发明的编码方法之间的比较。颜色由 B、G、Y 和 R 指示，其分别指示蓝色、绿色、黄色和红色。

[0051] 图 2 显示了表示以四个颜色对 6 个分析物（包括对照）检测的示意图。使用连接到荧光团 (B、G、Y、R；分别为蓝色、绿色、黄色和红色）和猝灭剂（标有“X”的椭圆形）的杂交探针检测分析物。对照序列和五个其它序列（登革热＝登革热病毒；结核病＝结核分枝杆菌 (Mycobacterium tuberculosis)；P17 ＝ HIV p17；疟疾＝恶性疟原虫 (Plasmodium falciparum)；和疱疹＝单纯性疱疹病毒 2) 均使用标有蓝色荧光团的探针检测。每个非对照分析物分别使用 1-3 个额外的探针检测。例如，登革热病毒的分析物使用三个具有绿色、黄色和红色荧光团的额外的探针检测；单纯性疱疹病毒 2 的分析物使用具有红色荧光团的额外的探针检测；等等。

[0052] 图 3 显示了如实施例 2 中描述得到的实验结果的色谱。

[0053] 图 4 显示了本发明的示例性的实施方案，其中使用计算机执行本文提供的方法的一个或更多个步骤。

[0054] 图 5 显示了检测光信号，例如荧光发射信号的波长和强度的两个不同的方法的示意图。

[0055] 图 6 显示了示例性的试剂盒的示意图。

[0056] 图 7 显示了如实施例 3 中描述得到的实验结果的色谱。

[0057] 图 8 显示了合理的结果（上图）和不合理的结果（下图）的示意图。

[0058] 发明详述

[0059] 由于几个理想的特性，包括与生化测定的相容性、荧光标记相对小的尺寸、缀合感兴趣的分子的简单的手段、可负担性、低毒性、稳定性、鲁棒性、利用廉价光学系统的可检测性、及与空间阵列相结合的能力，荧光检测已经是多路测定优选的技术。然而，标准的荧光具有广泛的发射光谱。因此，为了避免光谱重叠（即，为了保持光谱分辨率）仅相对少量数目的颜色（例如，4 至 6 种）通常在多路荧光测定中同时使用。

[0060] 多路荧光测定的传统编码方法一直以单一的颜色编码每个分析物；即，M ＝ N，其

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中 M 是可检测的分析物的数目，且 N 是光谱可分辨的荧光探针的数目。每当需要更高的多路因子时（即，M>N），荧光通常结合其它技术技术，如等分、空间阵列、或顺序处理。这些额外的处理步骤是劳动密集的，并经常需要相对昂贵和复杂的光学和机械系统（例如，光谱仪、机械化显微镜镜台、微流体、液滴生成器、扫描仪、及类似系统）。这种系统在某些环境中配置往往是不切实际的，特别是定点护理 (point-of care) 和低资源设置。因此，对可提供廉价的多路的手段，同时避免使用昂贵的额外的处理步骤的多路编码和解码方法有显著需求。

[0061] 本公开提供了用于样品中的多个分析物的检测的方法、系统、组合物和试剂盒。本文呈现了基于编码、分析和解码方法对分析物进行检测。在一些实例中，待检测的每个分析物被编码为信号（例如，强度）的值，其中值被分配，使得测定的结果明确地指示被测定的分析物存在或不存在。在其它实例中，待检测的每个分析物被编码为在信号的至少两个分量（例如，强度和波长）的每个中的值。信号的至少两个分量可以是正交的。类似地，如本公开中在其他地方更全面地描述的，可以使用多个正交信号，例如荧光信号和电化学信号的组合。分析物可以是任何合适的分析物，例如多核苷酸、蛋白质、小分子、脂质、碳水化合物、或其混合物。信号可以是任何合适的信号，例如电磁信号、光信号、荧光发射信号、电化学信号、化学发光信号、及其组合。信号的至少两个分量可以是任何合适的两个分量，例如振幅和频率，或强度和波长。

[0062] 编码分析物之后，样品被提供，其中样品包括或可包括编码的分析物中的至少一个。样品与在编码方案中对每个分析物指定的在分析物存在时、产生特定的信号的、一个或多个分析物特异性试剂接触。试剂可以是任何合适的试剂，其能够在其相应的分析物存在时产生这样的信号，例如，连接到荧光团和猝灭剂的寡核苷酸探针（例如，TAQMAN 探针）。如果试剂是连接到荧光团和猝灭剂的寡核苷酸探针，可执行产生信号的核酸扩增。

[0063] 添加试剂后，信号被定量。在一些情况下，通过测量信号的一个分量（例如，荧光强度），及基于用于编码每种分析物的存在的值、及信号的累积值，确定某些分析物存在和不存在，执行这种定量。

[0064] 在一些情况下，对样品累积地测量信号的至少两个分量（例如，强度和波长）。例如，这种测量可以通过测量在特定波长的强度，或特定波长范围内的强度执行。然后可以基于信号的至少两个分量中的每个分量的值与用于编码分析物的存在的值（即，在编码方案中的那些值）确定分析物的存在或不存在。

[0065] 编码可以以减少或消除通过该方法获得的可能的简并（例如，不明确的）结果的数目的方式执行。如本说明书中其他地方所描述的，特定编码方案的完整编码能力可被枚举，且某些潜在的分析物代码可从编码方案消除，以减少或消除简并性。同样地，编码方案可被设计成非简并的，使得简并性的减少或消除是没有必要的。可以构建解码矩阵以对应于累积信号测量结果的构成信号，将累积信号测量结果（例如，强度或在特定波长的强度）解释为某些分析物存在或不存在。

[0066] I. 定义

[0067] 本文所用的术语仅用于描述特定实施方案的目的，并不旨在限制。

[0068] 如本文所用，单数形式的“一 (a)”、“一 (an)”、和“该 (the)”除非上下文另有明确地指示，也旨在包括复数形式。此外，针对术语“包括 (including)”、“包括 (includes)”、“具

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有 (having)”、“具有 (has)”、“有 (with)”、“例如 (such as)”、或其变体的范围，在说明书和 / 或权利要求书中使用时，这些术语不是限制的，且旨在以类似术语“包括 (comprising)”的方式被包括。

[0069] 如本文所用的术语“约”，一般是指比特定用法的上下文中规定的数值增加 15％或减少 15％的范围。例如，“约 10”将包括从 8.5 到 11.5 的范围。

[0070] 如本文所用的术语“维”和“分量”，当涉及信号时，一般是指可被定量的信号的方面。例如，如果是由荧光分子产生的信号，其可以依据其波长（例如，第一维或第一分量）及其强度（例如，第二维或第二分量）被量化。

[0071] 如本文所用的术语“解码”，一般是指确定哪个分析物存在的方法，其基于累积信号和能够将累积信号转换为关于一个或更多个分析物存在或不存在的信息的编码方案或解码矩阵。

[0072] 如本文所用的术语“编码”，一般是指表示分析物的处理，使用包括信号的值（例如强度）、或在一个信号或多个信号的至少两个分量中的每个分量的值（如波长和强度）的代码。

[0073] 术语“连接到荧光团和猝灭剂的寡核苷酸探针”和“TAQMAN 探针”通常是指在多核苷酸扩增测定中用于检测分析物存在的水解探针。这些探针包括连接到荧光团和猝灭剂的寡核苷酸探针。只要猝灭剂和荧光团接近，猝灭剂就会猝灭由光源激发荧光团所发射的荧光。寡核苷酸探针的序列被设计为互补于分析物中存在的多核苷酸序列，并因此，能够与分析物中存在的多核苷酸序列杂交。寡核苷酸探针的杂交在包括引物的核酸扩增反应（例如，聚合酶链式反应）中执行。通过 DNA 聚合酶延伸引物后，聚合酶的 5’ 至 3’ 外切酶活性降解探针，释放荧光团和猝灭剂至介质中。荧光团和猝灭剂之间的接近被破坏，且来自荧光团的信号不再猝灭。因此，检测到的荧光的量是分析物存在的量的函数。如果没有分析物存在，探针将不与分析物杂交，且荧光团和猝灭剂将保持极为接近。很少信号或没有信号会产生。

[0074] 如本文所用的术语“正交”，一般是指可以独立地或近似独立地改变的信号的至少两个分量（如，波长和强度），或至少两个不同的信号（如，荧光发射信号和电化学信号）。例如，当使用荧光分子时，波长和强度被认为是正交的或近似正交的。除其它因素外，荧光发射的波长将取决于荧光分子的组合物，并且荧光的强度将取决于存在的分子的量。虽然波长和强度是可以近似独立地被改变的信号的两个分量的实例，但是本文描述的方法不限于可以独立地或近似独立地被改变的分量。也可以使用非独立地改变的信号的分量、或多个信号，只要足够良好地确定分量或信号的特征，以能够编码、测量和解码。例如，如果一个分量或信号中的变化影响另一个分量或信号中的变化，只要理解变化之间的关系，仍可以使用这两个分量或信号。

[0075] 如本文所用的术语“多核苷酸”、“寡核苷酸”、或“核酸”，在本文中用于指包括多个核苷酸的生物分子。示例性的多核苷酸包括脱氧核糖核酸、核糖核酸、及其合成的类似物，包括肽核酸。

[0076] 如本文所用的术语“探针”一般是指在特定的分析物存在下能够产生信号的试剂。探针通常具有至少两个部分：能够特异性识别分析物，或其一部分的部分；及能够在分析物，或其一部分存在下，产生信号的部分。如上文和本公开中其他地方描述的，探针可以是

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连接到荧光团和猝火剂的寡核苷酸探针。探针还可以是在分析物存在下，产生信号的任何试剂，如检测分析物的抗体，具有因抗体结合至分析物而发射或被猝灭的荧光标记。任何合适的探针可以以本公开中提出的方法使用，只要探针在分析物存在下产生可定量的信号。例如，探针的分析物特异性部分可以与酶耦合，在分析物存在下，所述酶转化不带电荷的底物为带电荷的产物，从而随着时间的推移提高了介质中的导电率。在此情况下，不同的分析物可以以不同比例通过耦合探针（如杂交探针或抗体）的分析物特异性部分至酶被编码。介质中增加的导电率的产生速率将对介质中存在的所有分析物累积。根据本文提供的方法，编码分析物能够将导电率测量结果转换为明确的（即，非简并的）的结果，提供了关于特定分析物存在或不存在的信息。类似地，探针可包括从底物产生化学发光产物的酶。化学发光的量可随后被用于编码特定分析物的存在。

[0077] II. 编码和解码方法

[0078] A. 传统荧光编码和解码方法

[0079] 确定分析物存在的常用的方法是使用 4 个光谱可分辨的荧光分子指示四个分析物的存在或不存在。图 1 的左手侧表示了这种方法的实例。图 1 的左手侧显示了编码方法，其中四个分析物（序列 1、序列 2、序列 3 和序列 4）每个分别由单一颜色（分别为蓝色、绿色、黄色和红色）编码。颜色表示连接到还包括猝灭剂的寡核苷酸探针的荧光团。在图 1 的左手侧所示的系统中，有四种不同的寡核苷酸探针，每种包括单一的荧光团（蓝色、绿色、黄色、或红色）和猝灭剂。分析物的存在或不存在是基于在特定的颜色中信号的存在或不存在确定的。

[0080] 图 1 的左手侧的图表显示了包含两个分析物：序列 1 和序列 3，的假定样品的强度对颜色。这些分析物的存在是基于蓝色信号（对应于序列 1）和黄色信号（对应于序列 3）的测量结果确定的。不存在序列 2 和序列 4 是通过不存在蓝色和红色信号指示的。

[0081] 表 1 显示了这种编码方案的二进制格式的解释。每个分析物被编码为在荧光信号的两个分量中的每个分量的值：(1) 颜色（也称为波长；或波长范围）和 (2) 的强度（由表中的数字指示）。例如，A（例如，序列 1）具有蓝色的颜色和 1 的强度；B（例如，序列 2）具有绿色的颜色和 1 的强度；等等。如本文所述的定量每个颜色范围内的信号的强度，例如，通过测量由带通滤波器确定的特定波长范围内的信号的强度。1000 的结果指示仅分析物 A 存在；1100 结果指示分析物 A 和 B 存在；等等。

[0082] 表 1. 具有四个探针的四个分析物的传统编码，每个探针具有单一颜色，且每个分析物一个探针。

[0083]

	蓝色	绿色	黄色	红色
A	1	0	0	0
B	0	1	0	0
C	0	0	1	0
D	0	0	0	1

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[0084] B. 每种分析物使用一种以上的颜色的编码方法

[0085] 上文描述的传统方法经受其由光谱可分辨的荧光团的数目限制的事实。更具体地，可检测的分析物的数目等于光谱可分辨的荧光团的数目。因此，分析物的数目仅可通过增加光谱可分辨的荧光团的数目增加。

[0086] 本公开提供了克服这种限制的方法。更具体地，在一些情况下，通过在编码过程中，利用信号的至少两个分量，本文描述的方法可用于检测每个荧光团一个以上的分析物。例如，使用本文提供的方法，每个荧光团可检测 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、或 50 个分析物。在一些情况下，本文提供的方法可用于检测，每个荧光团可检测至少 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、或 50 个分析物。在一些情况下，本文提供的方法可用于检测每个荧光团 1.5-2、2-4、1.5-4、4-6、2-6、6-10 个分析物。

[0087] 在一些情况下，本公开提供的方法可包括使用对照颜色。对照颜色可连接到一个或更多个探针，在样品中，所述探针结合阳性对照分析物，及待检测的每个分析物。如果在阳性对照分析物和待检测的每个分析物中出现相同的序列，也可以使用单一的对照探针。如果阳性对照分析物和待检测的每个分析物中不出现相同的序列，可以使用不同的探针，但每个探针仍可连接到对照颜色。

[0088] 例如，建立在上文描述的传统方法上，一种颜色（例如蓝色）可用于编码始终存在于样品中的对照分析物的存在。对照分析物可被加入到样品中，或者可以是固有地存在于样品中。剩余的颜色（例如，绿色、黄色、红色）可用于编码额外的分析物的存在。表 2 显示了一个这样的方法的实例。

[0089] 表 2. 以四个颜色且每个分析物最多两个颜色编码四个分析物（包括对照）。

[0090]

	蓝色	绿色	黄色	红色
对照 (p)	1	0	0	0
A	1	0	0	1
B	1	0	1	0
C	1	1	0	0

[0091] 在表 2 中，对照组 (p) 的存在由 1000 的结果指示。对照和分析物 A 的存在由 2001 的结果指示。对照和所有其它三个分析物的存在由 4111 的结果指示。对照颜色（蓝色）提供了测定正常运行的指示。蓝色颜色的强度报告了测试样品中存在的分析物的数目。当然，任何颜色都可以被用于对照颜色。本领域技术的一名人员将认识到，某些实际考虑可能使其优选使用一种颜色而不使用另一种颜色作为对照颜色。例如，如果一种颜色在特定的光学系统（或荧光团系统）中被更好地检测，那么可能实际上优选使用该颜色作为对照颜色。

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[0092] 表 2 中所示的编码方案使用一个对照颜色和一个额外的颜色（每个分析物最多 2 个探针）编码每个分析物。然而，如下所示，当每个分析物使用多达 4 个颜色时，可被编码的分析物的数目增加。表 3 显示了示例性的编码方案，其中每个分析物由最多 4 个颜色编码。表 3 所示的方案中，对照颜色（蓝色）在对照序列和其它七个分析物 (A-G) 的每个分析物的存在下产生。其它七个分析物的每个由一至三个额外的颜色的存在编码。颜色可能被不同的探针（例如，连接到荧光团和猝灭剂的寡核苷酸探针）或相同的探针（其可以具有多个荧光团和多个猝灭剂）所包含。

[0093] 表 3. 以四个颜色且每个分析物最多四个颜色编码八个分析物（包括对照）。

[0094]

	蓝色	绿色	黄色	红色
对照 (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

[0095] 在表 3 中，对照和所有其它七个分析物的存在由 8444 的结果指示。三个分析物 (A、B 和 C) 由两个颜色编码。三个分析物 (D、E 和 F) 由三个颜色编码，且一个分析物 (G) 由四个颜色编码。对照 (p) 的存在由 1000 的结果指示。对照和分析物 A 的存在由 2001 的结果指示。对照和分析物 G 的存在由 2111 的结果指示。这些结果表达每个颜色中信号的累积强度。例如，结果 2111 在蓝色通道中具有 2X 的信号强度，而结果 1000 在蓝色通道中仅具有 1X 的信号强度。

[0096] 使用表 3，依据颜色（蓝色、绿色、黄色、红色）和强度 (0-8)，每个可能的累积测定结果可被枚举。表 4 显示了通过基于在表 3 中所示的编码方法，对每个可能的累积测定结果进行枚举，并且根据在样品中存在的分析物提供每个测定的相应的解码结果，产生“解码矩阵”。通过基于编码方案枚举每个可能的累积测定结果，及根据在样品中存在的（或不存在的）分析物提供每个测定的相应的解码结果，可以产生用于本文描述的任何编码方案的类似的解码矩阵。在一些情况下，如以下所述，一个或更多个分析物可从编码方案除去，以减少或消除简并性。

[0097] 表 4. 表 3 所示的编码方法的解码矩阵。

[0098]

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累积测定结果				
蓝色	绿色	黄色	红色	存在的分析物
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

[0099]

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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

[0100]

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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

[0101] 使用两个假定构建的表 4 中提供了解码矩阵。首先，解码矩阵假定阳性对照 (P) 总是产生阳性的结果。第二，解码矩阵假定，每个颜色中，无论信号可来自哪个探针，强度都以与改变探针浓度相同的方式添加和衡量。这本质上意味着信号是加性的和数字的。这些假定所依据的条件可以通过适当准备测定得到满足。如在本文所提供的实施例中证明的，如果使用荧光信号，强度仅需近似是的加性的和数字的。

[0102] 表 4 允许荧光波长的四个范围中强度（即，每个颜色范围中的信号强度）的累积测量转换为样品中存在的相应的分析物。例如，4321 的结果指示存在 p、C、F 和 G，且其它分析物不存在。此结果被称为“合理”的结果，因为其在解码矩阵中出现。相比之下，4000 的结果，虽然可能测量到，但未在解码矩阵出现。更具体地，4000 的结果不能通过添加来自表 3 的对照代码和分析物代码的任意组合被实现。该结果被称为“不合理”的结果。不合理的结果可能指示测定故障。因此，对照 (p) 和解码矩阵提供了验证测定是否正常运行的手段。

[0103] 表 4 穷举了产生 4 个可分辨发射光谱（例如，颜色）的荧光团的任意组合。术语“秩 (rank)”用于描述检测的包括对照的分析物的数目。在上文提供的实例中，如果测定正常运行，那么秩等于蓝色信号的值。例如，秩 8 指示对照及所有其它七个分析物 (A-G) 存在。秩 2 指示存在对照及仅有的一个分析物。最低的秩是秩 1，这指示仅存在对照。在每个秩的可能性的数目可被枚举。例如，继续参考在表 3-4 中描述的编码和解码方法，对于秩 2 有 7 个可能性。在秩 3 的可能性的数目可以以 7 取 2 的组合计算或者说有 7！ /(5！ *2！) = 21 个可能性。更普遍地，可能性的数目是 N 取 K 的组合，即为 N！ /((N-K)！ *K！)。类似地，在秩 4、5、6、7 和 8，可能性的数目分别是 35、35、21、7 和 1。参照表 4 显示了表与理论预测一致。因此，表 4 是对表 3 中提供的编码方法的穷举的解码矩阵。

[0104] C. 减少或消除简并性

[0105] 如本文使用的术语“简并”和“简并性”，通常描述合理的结果是不明确的情况，因为依据分析物存在或不存在，其可以指示一个以上的可能性。例如，参照表 4，结果 5233 是简并的，因为它可以被解码为 pADFG 或 pBDEG。类似地，结果 4222 是简并的，因为它可以被解码为以下任一个 :pCDG、pAFG、pBEG、pDEF。相比之下，结果 3110 只能指示 pBC，且因此是非简并的。

[0106] 本公开提供了减少或消除简并性的方法，从而提高了通过其检测到分析物的置信度。在一个实施方案中，消除简并度的方法包括 :(i) 将待检测的每个潜在分析物编码为信号的值，并且可选地，编码为信号的至少两个分量的每个分量中的值 ;(ii) 枚举可以从编

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码方案获得的每个合理的结果；(iii) 鉴定是简并的每个合理的结果；及 (iv) 从编码方案消除至少一个潜在分析物（或潜在的分析物代码），其中消除该至少一个潜在的分析物减少或消除简并性。例如，参照表 3 中描述的编码方案，及在表 4 中描述的解码矩阵（枚举每个合理的结果），消除分析物 D、E 和 F 的任意两个，消除了简并性。消除分析物 D、E 和 F 中的任意一个不会消除简并性，但会减少它。

[0107] 继续参照表 3 中描述的编码方案，从编码方案消除分析物 D、E 和 F 的任意两个，产生仅使用 4 个颜色，以无简并性可以分析 6 个分析物（包括对照）的方案。相比之下，多路常规方法使用 4 种颜色将仅允许报告 3 个分析物和 1 个对照。因此，通过使用本文提供的方法可被分析的分析物的数目接近加倍。

[0108] 图 2 显示了本发明的示例性实施方案，其中使用 4 个颜色检测五个分析物和一个对照。分析物是来自登革热病毒（“登革热”）、结核分枝杆菌（“结核病”）、人类免疫缺陷病毒 (HIV)p17(P17)、疟原虫（“疟疾”）、和单纯性疱疹（“疱疹”）的核酸。在此实例中“颜色”是连接到还包括猝灭剂的寡核苷酸探针的荧光团。寡核苷酸探针通常将对不同的分析物不同，而颜色是相同的。例如，指定为探针 1 的所有探针具有蓝色颜色，但通常会具有不同的寡核苷酸序列。当然，如果互补序列在每个分析物中存在，指定为探针 1 的探针也可以具有相同的寡核苷酸序列。本公开中其他地方描述了用这些探针检测的机制。探针 1（蓝色）杂交包括对照的所有 6 个分析物。探针 2（绿色）杂交来自登革热病毒、分歧杆菌和 HIV p17 的分析物。探针 3（黄色）杂交来自登革热病毒和疟原虫的分析物。探针 4 杂交来自登革热病毒、分歧杆菌和单纯性疱疹的分析物。通过这些探针定义的编码方案被示于表 5。参照表 3，潜在的分析物 D(1011) 和 F(1110) 已经从编码方案消除。因此，表 5 中所示的编码方案是非简并的，且来自测定的每个合理的结果对应于样品中分析物的唯一的组合的存在或不存在。

[0109] 表 5. 如图 2 所示例的，用于检测登革热病毒、分枝杆菌、HIV、疟原虫和单纯性疱疹的编码方案。

[0110]

	蓝色	绿色	黄色	红色
对照 (p)	1	0	0	0
单纯性疱疹	1	0	0	1
疟原虫	1	0	1	0
HIV	1	1	0	0
分歧杆菌	1	1	0	1
登革热病毒	1	1	1	1

[0111] 上文所示的用于编码和解码的方法，包括用于消除简并性的方法，都同样地适用于使用额外的颜色的编码方法。例如，表 3 可以通过包括额外的颜色和额外的强度扩大（下面进一步描述）。然后如上所述，枚举可以从编码方案获得的每个合理的结果产生解码矩

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阵。可以为本公开中描述的任何编码方案构建类似于表 4 中提供的解码矩阵的解码矩阵。然后鉴定简并的合理的结果，且至少一个潜在的分析物代码从编码方案消除，以减少或消除简并性。消除简并性的方法可以使用在计算机可读介质上的软件、或使用执行该方法的硬件配置（例如，微芯片）执行。

[0112] 虽然简并性可由以上、及在本公开中其他地方描述的方法减少或消除，本公开还提供了通过设计的非简并的编码方案。例如，消除表 3 中描述的编码方案的简并性后，编码方案可无限地以非简并的方式扩大，其中非简并性是通过设计（参见例如，表 6 中示例的编码方案，下文更充分地描述）。类似地，本公开提供通过设计的完全非简并的编码方案，且因此不需要任何简并性减少或消除（参见例如，表 8 中示例的编码方案，下文更充分地描述）。因此，本公开提供的编码方案可能已经通过设计减少或消除了简并性，或通过设计是非简并的。

[0113] D. 使用一个以上的颜色和一个以上的强度的编码方法

[0114] 上文描述的编码方法（例如，表 3-5）可以通过允许由大于 1 的强度编码分析物被进一步扩大。例如，表 5 中提供的编码方案中的编码的每个分析物是由 1 或 0 的荧光强度编码的。在至少一个颜色中允许较高的信号强度值进一步增加了可以通过本公开中提供的任何方法编码的分析物的数目。在一些实例中，为了保持对照颜色的分析物计数能力，这些较高的强度值可被分配除了对照颜色的任何颜色。

[0115] 表 6 显示了利用四个颜色和多强度的示例性编码方案的最前面的三层。表 6 的第 1 层是表 3 的复制，显示了使用四个颜色的七个分析物和一个对照的编码。如上文描述的，潜在的分析物 D、E 和 F 的任何两个可从编码方案消除，以便产生非简并的编码方案。表 6 的第 1 层指示潜在的分析物 D(1011) 和 F(1110) 已经从编码方案消除，以消除简并性。因此，如上文描述的，表 6 的第 1 层所示的编码方案能够使用四个颜色确定五个分析物及一个对照的存在。

[0116] 表 6 的第 1 层的编码方案可通过允许任何颜色的强度增加，被扩大到第二层（层 2）。如上文描述的，为了保持对照的序列计数能力，对照颜色的强度可保持为 1。增加任何剩余三个颜色的强度将产生代码 100Y、10Y0、1Y00、10YY、1Y0Y、1YY0 和 1YYY，其中 Y>1。用于编码新的层的 Y 的最小值等于来自现有层的累积最大值加 1。如下文描述的，也可以使用大于 1 的值，以最大化强度之间的差异。

[0117] 因此，在此上下文中，术语“层”通常用于描述充分利用通过特定数目的没有简并性的第一值（例如，强度）和第二值（例如，颜色），提供编码能力的代码组。例如，表 6 的第 1 层充分利用通过没有简并性的四个颜色与每个颜色最多 1 个强度提供的编码能力。如表 6 所示，第 1 层，这将导致包括对照的六个编码的分析物。引入第二层，如上所述，Y 的最小值可被增加至等于根据现有层的累积最大结果加 1（或多于一）。在表 6 提供的实例中，蓝色（对照）颜色的强度被保持为 1，以保持这种颜色的序列计数能力。因此，第 2 层包括五个非简并的编码的可能性，其通过将绿色、黄色和红色通道中的强度增加至等于根据第 1 层的这些通道的每个通道中的累积最大结果加一获得。然后可以枚举层 2 的这些代码的所有可能性，且导致简并性的代码可被消除，或第 2 层可以通过设计，使用用于消除来自第 1 层的对应的代码的信息，制成非简并的。然后可通过添加根据类似的方法构建的第三层、或更多的层实现更大的编码容量。编码方案可具有无限数目的层，例如 1、2、3、4、5、6、7、8、

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9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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 或更多层。

[0118] 更具体地，参照表 6，第 2 层中的分析物 H 由 1004 编码。对照颜色的值保持为 1。红色颜色的值等于根据先前层的累积最大结果 (3) 加 1，或 4。同样地，分析物 I 和 J 由 1030 和 1400 分别编码。这些代码的组合用于编码其余四个分析物 (K-N)，如在第 1 层对分析物 D-G 完成的。这样完成了第 2 层。在第 2 层，潜在的分析物 K(1034) 和 M(1430) 的内容导致简并性。因此，这些分析物已从编码方案消除，以消除简并性。

[0119] 继续参照表 6，第三层 (层 3) 使用上文描述的相同的原理构建。分析物 O 用 1-0-0-16 编码。对照颜色的值仍然保持为 1。红色颜色的值等于根据先前层的累积最大结果 (15) 加 1、或 16。同样地，分析物 P 和 Q 分别由 1-0-9-0 和 1-16-0-0 编码。这些代码的组合用于编码其余四个分析物 (R-U)，如在第 1 层对分析物 D-G 及在第 2 层对分析物 K-N 完成的。在第 3 层，潜在的分析物 R(1-0-9-16) 和 T(1-16-9-0) 的内容导致简并性。因此，这些分析物已从编码方案消除，以消除简并性。

[0120] 表 6 中显示的三层的编码方案显示了使用四个颜色的 15 个分析物和一个对照的编码。通过增加更多的强度以产生额外的层和 / 或增加更多的颜色以在层内产生额外的编码能力，此编码方案可无限地扩大。如在本公开中描述的，减少或消除简并性的方法可以在添加每个强度和 / 或颜色时使用，以减少或消除简并的结果。

[0121] 更普遍地，表 6 中描绘的编码方案是表 3-5 中描绘的编码方案的非简并的、无限扩大。在第一层的累积测量的最大强度是 6。在第二层的累积测量的最大强度是 15。在第三层的累积测量的最大强度是 63，且以此类推。给出最大累积强度值 (F)，本编码方案中可用的层 (T) 的最大数目为 $T = \log_4(F+1)$。表 6 中描绘的编码方案提供了每层五个 (P 个) 非简并的代码。因此，代码的最大数目 $M = 5*\mathrm{Log}_4(F+1)$，或 $M = P*T$，其中，P 为每层的非简并的代码的数目，且 T 是层数。例如，给出 $F = 63$，对每层 5 个非简并的化码，代码 (即，分析物) 的最大数目为 15。此式不包括代码 1000，其在表 6 中保留为阳性对照。为了在分析物的总数中包括对照，任何人可简单地增加一，提供公式 $M = (P*T)+1$。

[0122] 本公开中提供的方法可用于无限扩大此编码方案。例如，通过利用不同的强度 (即，第一值) 和颜色 (即，第二值) 的组合，任何人可以通过改变每层的非简并的代码数目 (P) 和层的数目 (T)，编码任何数目 (M) 的分析物。例如，每层的非简并的代码的数目是 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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 或更多。类似地，层的数目可以是 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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 或更多。

[0123] 表 6. 使用四个颜色和多个强度编码 15 种分析物和 1 个对照。

[0124]

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层	分析物	B	G	Y	R	备注
1	对照（p）	1	0	0	0	
	A	1	0	0	1	
	B	1	0	1	0	
	C	1	1	0	0	

[0125]

	~~D~~	~~1~~	~~0~~	~~1~~	~~1~~	~~从编码方案中消除以消除简并性~~
	E	1	1	0	1	
	~~F~~	~~1~~	~~1~~	~~1~~	~~0~~	~~从编码方案中消除以消除简并性~~
	G	1	1	1	1	
累积最大结果		6	3	2	3	
2	H	1	0	0	4	
	I	1	0	3	0	
	J	1	4	0	0	
	~~K~~	~~1~~	~~0~~	~~3~~	~~4~~	~~从编码方案中消除以消除简并性~~
	L	1	4	0	4	
	~~M~~	~~1~~	~~4~~	~~3~~	~~0~~	~~从编码方案中消除以消除简并性~~
	N	1	4	3	4	
累积最大结果		11	15	8	15	
	O	1	0	0	16	
	P	1	0	9	0	
	Q	1	16	0	0	
	~~R~~	~~1~~	~~0~~	~~9~~	~~16~~	~~从编码方案中消除以消除简并性~~
	S	1	16	0	16	
	~~T~~	~~1~~	~~16~~	~~9~~	~~9~~	~~从编码方案中消除以消除简并性~~
	U	1	16	9	16	
累积最大结果						
3 个非简并层的累积最大结果		**16**	**63**	**26**	**63**	

[0126] 图 1 的右手侧显示了上文描述的方法的一个示例性的实例。更具体地，图 1 的右手侧显示了编码方法，其中 9 个或多于 9 个分析物（序列 1- 序列 9，等）中的每种个由至

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少两个颜色，且在每个颜色的强度变化进行编码。颜色表示连接到还包括猝火剂的寡核苷酸探针的荧光团。系统可设计成使得每个探针以单一荧光团标记，或每个探针以一个以上的荧光团标记。例如，对于分析物 H 的代码，在表 6 中是 1004。在红色通道中 4 的强度可通过使用包括 4 个红色荧光团的 H 特异性探针，或者通过使用每个包括单一的红色荧光团的 4 个 H 特异性探针实现。当然，产生在红色通道中 4 的结果的探针和荧光团的任意组合也同样合适，如每个含有 2 个红色荧光团的 2 个探针，及含有 1 个红色荧光团的 1 个探针和含有 3 个红色荧光团的 1 个探针，或仅仅是含有一个红色荧光团的一个探针，但以 4x 数量在反应混合物中存在。

[0127] 图 1 的右手侧描绘的编码方案可以如表 7 中所示。图 1 的右手侧所示的分析结果是 4112，或蓝色通道中强度为 4、绿色通道中为 1、黄色通道中为 1、且红色通道中为 2。使用如本文所述构建的解码矩阵，此结果被解码为指示序序列 1、序列 3、序列 4 和序列 5 存在。

[0128] 表 7. 图 1 的右手侧说明的编码方案。

[0129]

层	分析物	B	G	Y	R	备注
1	序列 1	1	0	0	0	
	序列 2	1	1	0	0	
	序列 3	1	0	1	0	
	序列 4	1	0	0	1	
	序列 5	1	1	0	1	
	序列 6	1	1	1	1	
	~~消除的潜在的序列~~	~~1~~	~~0~~	~~1~~	~~1~~	从编码方案中消除以消除简并性
	~~消除的潜在的序列~~	~~1~~	~~1~~	~~1~~	~~0~~	从编码方案中消除以消除简并性
累积最大结果		6	3	2	3	
2	序列 7	1	4	0	0	
	序列 8	1	0	3	0	
	序列 9	1	0	0	4	
等等-即，以如在表 6 中提供的继续						

[0130] E. 每个分析物使用一个颜色和一个强度，但在分析物中使用不同强度的编码方法

[0131] 在本文提供的一些方法中，每个分析物是由单一的颜色和强度的组合编码的。例如，在四色系统中，最前面的四个分析物可通过 1000、2000、4000 和 8000 编码。其后的四个分析物可通过 0100、0200、0400 和 0800 编码。分析物 9-12 和 13-16 将类似地分配，如表 8 所示。

[0132] 像表 6 中描述的编码方法，该编码方案通过构建在理论上是无限的、非简并的，且仅由测量信号使用的仪器的带宽限制。然而，此编码方案比表 6 中描述的编码方法能够在每单位带宽上定量更多的分析物。其原因在于，以最大的效率使用信号的可用的多路性，因为编码中利用了每个强度等级（即色谱中没有空缺；参见下文色谱的描述）。表 8 显示了

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该方法的一个实施方案，说明了基于四个颜色和强度 1、2、4 和 8 的四层编码。编码方案是非简并的，且如果所有 16 个分析物存在，结果是 15-15-15-15。在带宽利用率方面，这种编码方法比上面给出的编码方法更有效。然而，这种方法不具有第一个方案的校对能力，这是因为第一个方案中所有解码为合理的输出结果的结果都存在色谱中的空缺。

[0133] 表 8. 每个分析物使用一个颜色和一个强度，但在分析物中使用不同强度的编码方法的实例。

[0134]

层	分析物	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

[0135]

		15	15	15	15

[0136] 上文所示的，及表 8 中示例的方法，可通过引入额外的颜色和 / 或强度扩大。例如，可以使用 5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个颜色。也可以使用额外的强度等级，例如 16、32、64、128、255、512、1024、2048、4096、8192、16384、32768、65536、等等。该方法是可归纳的且可用于编码无限数目的分析物。每个分析物由单一颜色中的代码表示，其中，在该单一颜色中的代码的值等于所有先前值的总和加 1。例如，如果在特定的颜色中的第一代码包含 1，其后的代码是 2、4、8、16、32、64、128 等等。其它级数 (progression) 是可能的，但依据带宽的使用不会被认为有

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效的。然而，本领域的一个普通技术人员将认识到，当任何人希望最大化值之间的间隔时，可能希望较不有效的带宽使用。类似地，值 1 可从编码方案排除，例如，以最大化第一编码值和仪器的噪声之间的强度中的差异。例如，在一些情况下，编码方案可以以值 2 开始，其将为分析物代码提供为 2、4、8、16、32、64、128、等等的级数，而累积结果的级数将是 2、4、6、8、10、12、14、16、等等。这种方法在可能的累积结果中产生均匀的空缺 (gap)，相比 1、2、4、8、16、32、等等的分析物代码级数，允许对噪声更高的耐受，且其对应于 1、2、3、4、5、6、7、8、等等的累积结果级数。在这个实例中，测量中增加的鲁棒性以多路性的固定带宽中可用的代码的数目减少为代价。例如，如果仅有 63 个多路性状态能够被可靠地测量，那么从 1 开始的编码方案每个颜色提供 7 个代码，而从 2 开始的编码方案每个颜色提供 6 个代码。如果 4 个颜色可用，前者将提供 28 个代码，而后者将提供 24 个代码。总之，开始信号强度和级数（即，强度值之间的差异）两者均可以被衡量，以最大化对仪器的噪声的分辨能力，并最大化强度值之间的差异，由此增强区分不同实验结果之间的能力。

[0137] 如表 8 说明的编码方案通过设计为非简并的。虽然表 8 中说明的编码方案使用强度和颜色两者编码 16 个分析物的每个分析物，还可以通过简单地利用强度编码每个分析物。例如，给出表 8 中提供的 16 个分析物 (A-P)，单一颜色的编码方案编码全部 16 个分析物，可分别地分配值 1、2、4、8、16、32、64、128、256、512、1024、2048、4096、8192 和 16384 至分析物 A、B、C、D、E、F、G、H、I、J、K、L、M、N、O 和 P。这种编码方案通过设计为非简并的，且累积强度结果可以被明确地解码以指示分析物 A-P 的存在或不存在。这种编码方案能够检测 M 种分析物的存在或不存在，其中 $M = Log_2(F+1)$，且 F 是信号（例如，信号强度）的最大累积值。通过向这个信号添加第二分量（如颜色，如表 8 中描绘的），这个编码方案的容量可以增大到 $M = C*Log_2(F+1)$，其中 C 是在所述编码方案中使用的颜色数目。

[0138] 如贯穿此说明书中描述的，任何合适的值可以用于 C 或 F。例如，C 可以是至少约 2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多。F 可以是至少约 2、4、8、16、32、64、128、255、512、1024、2048、4096、8192、16384、32768、65536、131072、262144、524288、1048576、等等。如在本公开中其他地方描述的，F 的最初开始值和在其级数中的步长 (step) 也可以改变。

[0139] III. 分析物

[0140] 分析物可以是使用本发明的方法和组合物分析的任何合适的分析物，其中分析物能够与试剂相互作用，以便产生可被测量的具有至少两个分量的信号。分析物可以是天然存在的或合成的。分析物可以存在于使用本领域中已知的任何方法获得的样品中。在一些情况下，样品可以在其用于分析物的分析前被处理。本公开中提出的方法和组合物可在溶液相测定中使用，而不需要颗粒（如珠）或固体支持体。

[0141] 在一些情况下，分析物可以是多核苷酸，例如 DNA、RNA、肽核酸、及其任何杂合体 (hybrid)，其中多核苷酸包含脱氧核糖核苷酸和 / 或核糖核苷酸的任意组合。多核苷酸可以是单链或双链的，或同时含有双链或单链序列部分。多核苷酸可包含核苷酸或碱基的任意组合，包括，例如，尿嘧啶、腺嘌呤、胸腺嘧啶、胞嘧啶、鸟嘌呤、肌苷、黄嘌呤、次黄嘌呤、异胞嘧啶、异鸟嘌呤及其任意核苷酸衍生物。如本文所用，术语“核苷酸”可包括核苷酸和核苷，以及核苷和核苷酸类似物，及修饰 (modified) 的核苷酸，包括合成的种类和天然

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存在的种类两者。多核苷酸可以是针对其可产生如本文描述的一种或更多种试剂（或探针）的任何合适的多核苷酸，包括但不限于 cDNA、线粒体 DNA(mtDNA)、信使 RNA(mRNA)、核糖体 RNA(rRNA)、转移 RNA(tRNA)、核 RNA(nRNA)、小干扰 RNA(siRNA)、小核 RNA(snRNA)、小核仁 RNA(snoRNA)、小卡哈尔体特异性 RNA(small Cajal body-specific RNA，scaRNA)、微小 RNA(miRNA)、双链 (dsRNA)、核酶、核糖开关 (riboswitch) 或病毒 RNA。多核苷酸可被包含在任何合适的载体中，例如质粒、粘粒、片段、染色体、或基因组。

[0142] 基因组 DNA 可从天然存在的或遗传修饰的生物体，或从人工地或合成地产生的基因组获得。包括基因组 DNA 单独分析物可从任何来源，且使用本领域中已知的任何方法获得。例如，基因组 DNA 可以利用或不利用扩增被分离。扩增可包括 PCR 扩增、多重置换扩增 (MDA)、滚环扩增和其它扩增方法。基因组 DNA 也可以通过克隆或重组的方法获得，例如那些涉及质粒和人工染色体的方法或其它的常规方法（参见 Sambrook 和 Russell，Molecular Cloning:A Laboratory Manual.，出处同上）。多核苷酸可使用本领域已知的其它方法分离，例如如在 Genome Analysis:A Laboratory Manual Series（卷 I-IV）或 Molecular Cloning:A Laboratory Manual 中公开的。如果分离的多核苷酸是 mRNA，其可以使用常规技术被逆转录 (reverse transcribe) 为 cDNA，如 Sambrook 和 Russell，Molecular Cloning:A Laboratory Manual，出处同上，中描述的。

[0143] 分析物可以是蛋白质、多肽、脂质、碳水化合物、糖、小分子、或可以以本文提供的方法和组合物检测的任何其它合适的分子。分析物可以是酶或其它蛋白质。分析物可以是药物或代谢物（例如，抗癌药物、化学治疗药物、抗病毒药物、抗生素药物、或生物制剂 (biologic)）。分析物可以是任何分子，如辅因子、受体、受体配体、激素、细胞因子、血液因子、抗原、类固醇、或抗体。

[0144] 分析物可以是来自任何病原体的任何分子，病原体例如病毒、细菌、寄生虫、真菌、或朊病毒（例如，PrP^{Sc}）。病毒的实例包括来自以下科的那些，腺病毒科、黄病毒科、嗜肝病毒科、疱疹病毒科、正粘病毒科、乳多空病毒科、副粘病毒科、小核糖核酸病毒科、多瘤病毒科、逆转录病毒科、弹状病毒科、及披膜病毒科。病毒的具体实例包括腺病毒、星状病毒、博卡病毒、BK 病毒、柯萨奇病毒、巨细胞病毒、登革热病毒、埃博拉病毒、肠病毒、EB 病毒、猫白血病病毒、肝炎病毒、甲型肝炎病毒、乙型肝炎病毒、丙型肝炎病毒、丁型肝炎病毒、戊型肝炎病毒、单纯性疱疹病毒 (HSV)、HSV 1 型、HSV 2 型、人免疫缺陷病毒 (HIV)、HIV 1 型、HIV 2 型、人类乳头瘤病毒 (HPV)、HPV 1 型、HPV 2 型、HPV 3 型、HPV4 型、HPV 6 型、HPV 10 型、HPV 11 型、HPV16 型、HPV18 型、HPV 26 型、HPV 27 型、HPV 28 型、HPV 29 型、HPV 30 型、HPV 31 型、HPV 33 型、HPV 34 型、HPV 35 型、HPV 39 型、HPV 40 型、HPV 41 型、HPV 42 型、HPV 43 型、HPV 44 型、HPV 45 型、HPV 49 型、HPV 51 型、HPV 52 型、HPV 54 型、HPV 55 型、HPV 56 型、HPV 57 型、HPV 58 型、HPV 59 型、HPV 68 型、HPV 69 型、流感病毒、JC 病毒、马尔堡病毒、麻疹病毒、腮腺炎病毒、诺沃克病毒、细小病毒、脊髓灰质炎病毒、狂犬病病毒、呼吸道合胞病毒、逆转录病毒、鼻病毒、轮状病毒、风疹病毒、天花病毒、牛痘病毒、西尼罗河病毒和黄热病病毒。

[0145] 细菌的实例包括来自以下属的那些，博德特氏菌属 (Bordetella)、疏螺旋体属 (Borrelia)、布鲁氏菌属 (Brucella)、弯曲杆菌属 (Campylobacter)、衣原体属 (Chlamydia)、梭菌属 (Clostridium)、棒状杆菌属 (Corynebacterium)、肠球菌

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属 (Enterococcus)、埃希氏菌属 (Escherichia)、弗朗西斯氏菌属 (Francisella)、嗜血杆菌属 (Haemophilus)、螺杆菌属 (Helicobacter)、军团菌属 (Legionella)、钩端螺旋体属 (Leptospira)、李斯特菌属 (Listeria)、分枝杆菌属 (Mycobacterium)、支原体属 (Mycoplasma)、奈瑟菌属 (Neisseria)、假单胞菌属 (Pseudomonas)、立克次氏体属 (Rickettsia)、沙门氏菌属 (Salmonella)、志贺氏菌属 (Shigella)、葡萄球菌属 (Staphylococcus)、链球菌属 (Streptococcus)、密螺旋体属 (Treponema)、弧菌属 (Vibrio) 和耶尔森氏菌属 (Yersinia)。细菌的具体实例包括副百日咳博德特氏菌 (Bordetella par apertussis)、百日咳博得特氏菌 (Bordetella pertussis)、伯氏疏螺旋体 (Borrelia burgdorferi)、流产布鲁氏菌 (Brucella abortus)、犬布鲁氏菌 (Brucella canis)、羊布鲁氏菌 (Brucella melitensis)、猪布鲁氏菌 (Brucella suis)、空肠弯曲杆菌 (Campylobacter jejuni)、肺炎衣原体 (Chlamydia pneumoniae)、鹦鹉热衣原体 (Chlamydia psittaci)、沙眼衣原体 (Chlamydia trachomatix)、肉毒梭菌 (Clostridium botulinum)、艰难梭菌 (Clostridium difficile)、产气荚膜梭菌 (Clostridium perfringens)、破伤风梭菌 (Clostridium tetani)、白喉棒状杆菌 (Corynebacterium diphtheriae)、粪肠球菌 (Enterococcus faecalis)、屎肠球菌 (Enterococcus faecium)、大肠杆菌 (Escherichia coli)、土拉弗朗西斯菌 (Francisella tularensis)、流感嗜血杆菌 (Haemophilus influenzae)、幽门螺旋杆菌 (Helicobacter pylori)、嗜肺军团菌 (Legionella pneumophila)、问号钩端螺旋体 (Leptospira interrogans)、单增李斯特菌 (Listeria monocytogenes)、麻风分枝杆菌 (Mycobacterium leprae)、结核分枝杆菌 (Mycobacterium tuberculosis)、溃疡分枝杆菌 (Mycobacterium ulcerans)、肺炎支原体 (Mycoplasma pneumoniae)、淋球奈瑟菌 (Neisseria gonorrhoeae)、脑膜炎奈瑟菌 (Neisseria meningitidis)、铜绿假单胞菌 (Pseudomonas aeruginosa)、立克次氏体 (Rickettsia rickettsii)、猪霍乱沙门氏菌 (Salmonella choleraesuis)、都柏林沙门氏菌 (Salmonella dublin)、肠炎沙门氏菌 (Salmonella enteritidis)、伤寒沙门氏菌 (Salmonella typhi)、鼠伤寒沙门氏菌 (Salmonella typhimurium)、宋内氏志贺菌 (Shigella sonnei)、金黄色葡萄球菌 (Staphylococcus aureus)、表皮葡萄球菌 (Staphylococcus epidermidis)、腐生葡萄球菌 (Staphylococcus saprophyticus)、无乳链球菌 (Streptococcus agalactiae)、肺炎链球菌 (Streptococcus pneumoniae)、化脓性链球菌 (Streptococcus pyogenes)、苍白密螺旋体 (Treponema pallidum)、霍乱弧菌 (Vibrio cholerae)、鼠疫耶尔森氏菌 (Yersinia pestis) 和小肠结肠炎耶尔森菌 (Yersinia enterocolitica)。

[0146] 寄生虫的实例包括来自以下属的那些，棘阿米巴属 (Acanthamoeba)、巴贝虫属 (Babesia)、巴拉姆西属 (Balamuthia)、肠袋属虫 (Balantidium)、芽囊原虫属 (Blasocystis)、隐孢子虫属 (Cryptosporidium)、双核阿米巴属 (Dientamoeba)、内阿米巴属 (Entamoeba)、贾第虫属 (Giardia)、等孢球虫属 (Isospora)、利什曼原虫属 (Leishmania)、耐格里虫属 (Naegleria)、虱属 (Pediculus)、疟原虫属 (Plasmodium,)、鼻孢子虫属 (Rhinosporidium,)、肉孢子虫属 (Sarcocystis)、血吸虫属 (Schistosoma)、弓形虫属 (Toxoplasma)、滴虫属 (Trichomonas) 和锥虫属 (Trypanosoma)。寄生虫的具体实例包括分歧巴贝虫 (Babesia divergens)、双芽巴贝虫 (Babesia bigemina)、马巴贝虫

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(Babesia equi)、田鼠巴贝虫(Babesia microfti)、邓肯巴贝虫(Babesia duncani)、狒狒巴拉姆西阿米巴(Balamuthia mandrillaris)、结肠小袋纤毛虫(Balantidium coli)、脆弱双核阿米巴(Dientamoeba fragilis)、痢疾阿米巴(Entamoeba histolytica)、贾第鞭毛虫(Giardia lamblia)、贝利等孢球虫(Isospora belli)、福氏耐格里阿米巴(Naegleria fowleri)、人虱(Pediculus humanus)、恶性疟原虫(Plasmodium falciparum)、诺氏疟原虫(Plasmodium knowlesi)、三日疟原虫(Plasmodium malariae)、卵形疟原虫(Plasmodium ovale)、间日疟原虫(Plasmodium vivax)、鼻孢子虫(Rhinosporidium seeberi)、牛人肉孢子虫(Sarcocystis bovihominis)、猪人肉孢子虫(Sarcocystis suihominis)、曼氏血吸虫(Schistosoma mansoni)、弓形虫(Toxoplasma gondii)、阴道毛滴虫(Trichomonas vaginalis)、布氏锥虫(Trypanosoma brucei)、和克氏锥虫(Trypansoma cruzi)。

[0147] 真菌的实例包括来自以下属的那些,鳞质霉属(Apophysomyces)、曲霉属(Aspergillus)、芽生菌属(Blastomyces)、念珠菌属(Candida)、枝孢霉属(Cladosporium)、球孢子菌属(Coddidioides)、隐球菌属(Cryptococcos)、突脐孢属(Exserohilum)、镰刀菌属(Fusarium)、组织胞浆菌属(Histoplasma)、毕赤酵母属(Pichia)、肺囊虫属(Pneumocystis)、酵母属(Saccharomyces)、孢子丝菌属(Sporothrix)、葡萄穗霉属(Stachybotrys)和毛癣菌属(Trichophyton)。真菌的具体实例包括烟曲霉(Aspergillus fumigatus)、黄曲霉(Aspergillus flavus)、棒曲霉(Aspergillus clavatus)、皮炎芽生菌(Blastomyces dermatitidis)、白色念珠菌(Candida albicans)、粗球孢子菌(Coccidioides immitis)、新型隐球菌(Cryptococcus neoformans)、尖角突脐孢(Exserohilum rostratum)、轮状镰刀菌(Fusarium verticillioides)、荚膜组织胞浆菌(Histoplasma capsulatum)、杰氏肺囊虫(Pneumocystis jirovecii)、申克孢子丝菌(Sporothrix schenckii)、黑葡萄穗霉(Stachybotrys chartarum)和须癣毛癣菌(Trichophyton mentagrophytes)。

[0148] 在一些情况下,本公开中所提供的方法可以用于检测在上文、或者在说明书的其它地方描述的分析物中的任何一个。在一些情况下,本公开中提供的方法可以用于检测在上文、或在说明书的其它地方描述的分析物的组合(panel)。例如,组合可包括选自由任意2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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或更多个在上文或在说明书中的其它地方描述的分析物所组成的组的分析物。

[0149] 分析物可从任何合适的位置获得,包括从生物体、完整细胞、细胞制剂和来自任何生物体、组织、细胞、或环境的无细胞组合物。分析物可从环境样品、活检、抽吸物、福尔马林固定的包埋组织、空气、农业样品、土壤样品、石油样品、水样、或灰尘样品中获得。在一些情况下,分析物可以从体液获得,其可能包括血液、尿液、粪便、血清、淋巴液、唾液、粘膜分泌物、汗液、中枢神经系统流体、阴道液、或精液。分析物也可以由制造的产品获得,如化妆品、食品、个人护理产品、等等。分析物可以是实验操作的产物,包括重组克隆、多核苷酸扩增、聚合酶链式反应(PCR)扩增、纯化方法(如基因组DNA或RNA的纯化)和合成反应。

[0150] 在每个多路测定中,可以检测多于一种类型的分析物。例如,多核苷酸、蛋白质、多肽、脂质、碳水化合物、糖、小分子、或任何其它合适的分子可以同时在相同的多路测定中以合适的试剂检测。可在相同的时间检测分析物的任意组合。

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[0151] 分析物的检测可对任何合适的应用有用，应用包括科研、临床、诊断、预后、法医和监测应用。典型应用包括遗传性疾病的检测、遗传指纹的鉴定、感染性疾病的诊断、基因的克隆、亲子测试、刑事鉴定、系统发育、生物反恐、环境监测、及 DNA 计算。例如，分析物可以指示疾病或状况。分析物可以用来做出治疗决定，或以评估疾病的状态。分析物的存在可以指示具有特定病原体感染或任何其它疾病如癌症、自身免疫性疾病、心肺病、肝病、消化道疾病、等等。因此本文提供的方法可以用于做出诊断并基于该诊断做出临床决定。例如，指示从受试者采集的样品中细菌多核苷酸的存在的结果可能导致以抗生素治疗受试者。

[0152] 在一些情况下，本发明的方法和组合物可用于检测至少 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或 1000 个分析物。在一些情况下，本发明的方法和组合物可用于检测 7-50、8-40、9-30、10-20、10-15、8-12、或 7-12 个分析物。

[0153] 在一些情况下，本公开提供在单一样品体积中能够明确地检测处于存在或不存在的任意组合的 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或 1000 个分析物中的每个分析物的存在或不存在的测定而无需固定化、分离、质谱法、或解链曲线分析。在一些情况下，本公开提供在单一样品体积中能够明确地检测处于存在或不存在的任意组合的至少 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或 1000 个分析物中的每个分析物的存在或不存在的测定而无需固定化、分离、质谱法、或解链曲线分析。在一些情况下，本公开提供在单一样品体积中能够明确地检测处于存在或不存在的任意组合的少于 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或 1000 个分析物的存在或不存在的测定而无需固定化、分离、质谱法、或解链曲线分析。

[0154] IV. 信号

[0155] 本公开中提出的方法可以任何可定量的信号进行使用。在一些情况下，本公开提供了使用信号的单一分量（例如，强度），以非简并性编码无限数目的目标的编码方案和方法。例如，如在上文和在实施例 3 中描述的，编码方案可依赖于信号强度的多路性不考虑颜色。虽然荧光探针已被用于说明此原理，但是如在本公开中其他地方描述的，所述编码方案同样适合任何其它方法，所述其它方法提供了可定量的信号，包括电化学信号和化学发光信号。

[0156] 本公开提出的方法还可以利用在至少二个维度中的信号测量结果，也被称为信号的至少两个分量的测量结果。例如，相比于上文段落中描述的、依赖于信号强度以区分分析

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物的编码方案，利用信号的至少两个分量（例如，颜色和强度）允许每个信号强度带宽的单位产生更多不同的代码。当利用信号的至少两个分量时，可获得单一样品体积的至少两个分量的累积测量结果。例如，在表 5 描述的编码方案中，每个分析物的存在导致在 4 个颜色（信号的第二分量）的每个颜色上的特定的强度（信号的一个分量）。这些构成信号的组合导致了可以通过测量在每个波长或波长范围内的强度来测量累积信号。然后可使用相应的编码方案或解码矩阵，将累积测量结果转换为确定分析物存在或不存在。

[0157] 在一些情况下，可定量的信号包括具有频率（波长）和振幅（强度）两者的波形。信号可以是电磁信号。电磁信号可以是声音、无线电信号、微波信号、红外信号、可见光信号、紫外光信号、x 射线信号、或 γ 射线信号。在一些情况下，电磁信号可以是荧光信号，例如可以依据波长和强度表征的荧光发射光谱。

[0158] 在本公开的某些部分，信号依据荧光信号被描述和示例。这不意味着是限制性的，且本领域的一个普通技术人员将容易地认识到适用于荧光信号的测量的原理也适用于其它信号。例如，类似荧光信号，上文描述的任何电磁信号都可以依据波长和强度表征。荧光信号的波长也可以依据颜色描述。颜色可以通过基于在特定波长或波长范围测量强度确定，例如，通过确定在不同波长荧光的强度分布，和 / 或通过利用带通滤波器以确定特定波长范围内的荧光强度来进行确定。这样的带通滤波器通常用于各种实验室仪器，包括定量 PCR 器。强度可以以光电检测器测量。许多波长可被称为“信道”。

[0159] 在一些情况下，本公开中提供的方法可以用于任何信号，其中累积信号以相同的颜色、频率、吸收带、等等的构成信号衡量。然而，累积信号不需要是数字信号或随构成信号的数目和强度线性调节 (scale)。例如，如果测量的物理原理是吸收，那么由于 Beer-Lambert 定律的指数性质，累积衰减是构成衰减的乘积而构成浓度是加性的。然后累积衰减的对数将随在每个吸收带（如果使用荧光检测，相当于颜色）上的构成浓度线性调节。因此，本发明的方法是可以应用的。本发明的方法也可用于化学发光信号和电化学信号。

[0160] 信号的数目，及测量的维数或分量的数目，也可以扩大到超出本发明的示例性的实施方案中所示的数目，导致多路能力的扩大。本公开中提供的示例性实施方案利用了利用具有测量的一个或两个分量 :波长和 / 或强度的荧光信号所构造的编码方案。可被编码的分析物的数目可以通过增加波长和 / 或强度的数目而增加。可被编码的分析物的数量，也可以通过增加信号的数目而增加，例如通过组合荧光信号与电化学信号或 FRET 信号（荧光共振能量转移）而增加。

[0161] 在一些情况下，信号的两个以上的分量可被测量。例如，信号的 3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、18、20 个或更多个分量可被测量。信号的至少 2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、18、或 20 个分量可被测量。信号的至少 2 个，但少于 3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、18、或 20 个分量可被测量。在一些情况下，信号的 2-3、2-4、2-5、2-6、3-5、3-6、3-8、或 5-10 个分量可被测量。这些额外的分量可包括动力分量，例如信号衰减的速率和光漂白速率。

[0162] 如果采用荧光信号，可被编码的分析物的数目，可以进一步通过利用额外的荧光团扩大。例如，可以使用 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、

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48、49、50、或更多个荧光团。在一些情况下，可以使用至少 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或 50 个荧光团。在一些情况下，可以使用少于 2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或 50 个荧光团。在一些情况下，可以使用 4-6、4-7、4-8、4-9、4-10、5-6、5-7、5-8、5-9、5-10、10-15、或 10-20 的荧光团。

[0163] 通常，可被编码的分析物的数目，可以进一步通过利用来自信号的第一分量的值或值的范围的额外的第一值（例如，强度）扩大。例如，可以使用 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个来自信号的第一分量的值或值的范围的第一值。在一些情况下，可以使用至少 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个来自信号的第一分量的值或值的范围的第一值。在一些情况下，可以使用少于 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或 50 个来自信号的第一分量的值或值的范围的第一值。在一些情况下，可以使用 4-20、4-6、4-7、4-8、4-9、4-10、5-6、5-7、5-8、5-9、5-10、10-15、或 10-20 的来自信号的第一分量的值或值的范围的第一值。

[0164] 在实例中，其中强度是被定量的信号的分量，例如当利用荧光信号时，分析物的存在可以使用多个强度或强度范围编码。例如，编码方案可以利用 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个强度或强度范围。编码方案可以利用至少 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、20、21、22、23、24、25、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、或更多个强度或强度范围。编码方案可以利用少于 2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或 50 个强度或强度范围。在一些情况下，编码方案可利用 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 或 5-10 个强度或强度范围。

[0165] 可被编码的分析物的数目，可以进一步通过利用来自信号的第二分量的值或值的范围的额外的第二值（例如，波长）扩大。例如，可以使用 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个来自信号的第二分量的值或值的范围的第二值。在一些情况下，可以使用至少 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个来自信号的第二分量的值或值的范围的第二值。在一些情况下，可以使用少于 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、

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46、47、48、49、50、或更多个来自信号的第二分量的值或值的范围的第二值。在一些情况下，可以使用 4-20、4-6、4-7、4-8、4-9、4-10、5-6、5-7、5-8、5-9、5-10、10-15、或 10-20 个来自信号的第二分量的值或值的范围的第二值。

[0166] 在其中波长是被定量的信号的分量的实例中，例如当利用荧光信号时，分析物的存在可以使用多种波长或波长范围编码。例如，可以使用 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个波长或波长范围。在一些情况下，可以使用至少 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个波长或波长范围。在一些情况下，可以使用少于 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或 50 个波长或波长范围。在一些情况下，可以使用 4-20、4-6、4-7、4-8、4-9、4-10、5-6、5-7、5-8、5-9、5-10、10-15、或 10-20 的波长或波长范围。

[0167] 在一些情况下，当简并性被消除，本发明的方法能够检测在单一体积中的至少 6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个分析物的存在或不存在，当用于在体积中产生信号的每个试剂仅产生一个第二值（例如，每个试剂，仅发射一个波长的光）时。

[0168] 在其它情况下，当简并性被消除，本发明的方法能够检测在单一体积中的至少 7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个分析物的存在或不存在，总共在该体积中使用了四个第二值（例如，共四个波长或波长的范围，其可能通过使用四个光谱可分辨的荧光团实施）。

[0169] 如在整个说明书中描述的，本文提供的测定利用对样品的累积测量结果。累积测量结果可以是，例如，强度值的单一测量结果，或在一个或更多个波长或波长的范围的强度值的测量结果。可以获得多个累积测量结果。例如，强度可以在 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个波长或波长范围测量。强度可以在至少 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个波长或波长范围测量。强度可以在少于 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或 50 个波长或波长范围测量。

[0170] 更普遍地，可获得关于信号的任何可定量的分量，以及关于在信号的另一个可定量的分量的信号的任何可定量的分量的累积测量结果。例如，信号的至少一个第一分量可以 在 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个信号的第二分量测量。信号的至少一个第一分量可以在至少 1、2、3、4、5、6、7、8、9、10、11、

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12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个信号的第二分量测量。信号的至少一个第一分量可以在少于 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或 50 个信号的第二分量测量。

[0171] 如本公开明显显示的，待检测的每个分析物可被编码为利用任意数目的信号的合适的分量，或任意数目的信号的代码。例如，待检测的每个分析物可以被编码在 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个信号的分量或信号中。在一些情况下，待检测的每个分析物可以被编码在至少 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个信号的分量或信号中。在一些情况下，待检测的每个分析物可以被编码在少于 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或 50 个信号的分量或信号中。在一些情况下，可以使用 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、或 10-20 的信号的分量或信号编码每个分析物。在编码方案中每个分析物可以由相同数目的信号的分量，或信号，或不同数目的信号的分量，或信号编码。

[0172] A. 探针、引物、荧光团、和猝灭剂

[0173] 本公开中提供的一些方法利用在分析物存在时产生信号的试剂。任何合适的试剂可用于本发明。通常，试剂将具有分析物特异性成分，及在分析物存在时产生信号的分量。在一些情况下，这些试剂被称为探针。探针可以是杂交探针。杂交探针可以是连接到荧光团和猝灭剂（例如，TAQMAN 探针）的寡核苷酸探针。

[0174] 本发明的方法可使用一种或更多种试剂或探针以检测每个分析物的存在或不存在。例如，可以使用 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个探针以检测每个分析物的存在或不存在。在一些情况下，可以使用至少 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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 个探针，以检测每个分析物的存在或不存在。在一些情况下，可以使用少于 2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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 个探针以检测每个分析物的存在或不存在。在一些情况下，用于检测每个分析物的存在或不存在的探针的数目是 1-10、2-9、2-8、2-7、2-6、2-5、2-4、1-3、或 1-4。

[0175] 在一些情况下，样品与 4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个探针接触，以检测所有分析物的存在或不存在。在一些情况下，样品与至少 4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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 或更多个探针

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接触，以检测所有分析物的存在或不存在。在一些情况下，样品与少于4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个探针接触，以检测所有分析物的存在或不存在。在一些情况下，样品所接触的用以检测所有分析物的存在或不存在的探针的数目是4-50、4-40、4-30、4-20、5-15、5-10、或3-10。

[0176] 如上文描述的，连接到荧光团和猝灭剂的寡核苷酸探针可用于检测多核苷酸扩增测定中分析物的存在。只要猝灭剂和荧光团接近，猝灭剂就猝灭由光源激发荧光团所发射的荧光。寡核苷酸探针的序列被设计为互补于分析物中存在的多核苷酸序列，并因此，能够与分析物中存在的多核苷酸序列杂交。在包括引物的核酸扩增反应（例如，聚合酶链式反应）中可执行寡核苷酸探针的杂交。通过DNA聚合酶延伸引物后，聚合酶的5’至3’外切酶活性降解探针，释放荧光团和猝灭剂至介质中。荧光团和猝灭剂之间的接近被破坏，且来自荧光团的信号不再猝灭。因此，检测到的荧光的量是分析物存在的量的函数。如果没有分析物存在，探针将不与分析物杂交，且荧光团和猝灭剂将保持极为接近。很少信号或没有信号会产生。

[0177] 寡核苷酸探针的每个探针可具有一个或多个荧光团和猝灭剂。例如，在一些实施方案中，寡核苷酸探针可包括1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个荧光团。寡核苷酸探针可包括至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个荧光团。寡核苷酸探针可包括少于2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个荧光团。

[0178] 寡核苷酸探针可包括1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个猝灭剂。寡核苷酸探针可包括至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个猝灭剂。寡核苷酸探针可包括少于2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个猝灭剂。

[0179] 探针和猝灭剂至探针的连接可以在相同的反应，或在系列的反应中执行。可以执行一系列的反应以使用至少一种荧光团标记探针，且反应产物可被混合以产生具有不同荧光团的探针的混合物。

[0180] 如果寡核苷酸探针包括两个或更多个荧光团，这些荧光团可以被布置为允许Förster（荧光）共振能量转移(FRET)在荧光团之间发生。简单地说，FRET是在荧光团之间的能量转移的机制。使用基于FRET的探针允许一个激发源通过单一的荧光团的激发，产生两个不同的荧光发射信号。例如，本文描述的方法可使用配对的探针，其中在配对中的一个探针连接到荧光团和猝灭剂，且第二探针连接到两个荧光团（足够接近用于FRET发生）和猝灭剂。可以使用提供用于FRET的荧光团和猝灭剂任意组合。这种方法使得可以与激发源使用的荧光探针的数目加倍，这是因为单一的激发源可用于产生两个信号：一个来自

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具有一个荧光团的探针，且一个来自具有足够接近用于FRET发生的两个荧光团的探针。例如，使用表8中描述的编码方法，通过将表8中的每个探针与对应的FRET探针配对，明确地可检测的分析物的数目可从16增加到32。

[0181] 引物可以是对特定分析物特异性的，且能够扩增与探针互补的区域。在一些情况下，使用的引物的配对的数目等于探针的数目。在其它情况下，使用的探针的数目可超过使用的引物配对的数目。在又另一些情况下，使用的引物配对的数目可超过使用的探针的数目。在一些情况下，待分析的样品可与1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个引物的配对接触。在一些情况下，待分析的样品可与至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个引物的配对接触。在一些情况下，待分析的样品可与少于2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个引物的配对接触。在一些情况下，引物的配对的数目是2-10、3-15、4-20、3-10、4-10、5-10、6-8、或6-10。

[0182] 引物可扩增不同数目的杂交探针在其中杂交的多核苷酸的区域。例如，引物的至少一个配对可以扩增与至少2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个杂交探针互补的区域。在一些情况下，引物的所有所述配对可以扩增与至少2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个杂交探针互补的区域。

[0183] 在一个实例中，第一探针可以以荧光团5'-荧光素酰胺(5'-FAM)和猝灭剂黑洞猝灭剂1(BHQ-1)标记。第二探针可以以极为接近（例如，连接到）青色素5.5(Cy5.5)和猝灭剂黑洞猝灭剂3(BHQ-3)的5'-FAM标记。根据经由聚合酶的核酸酶活性探针的消化，从单一激发波长（例如，470nm）产生两个荧光信号。来自第一探针的荧光团在大约520nm发出荧光。在第二探针上的荧光团经受FRET。供体荧光团FAM通过激发光源（例如，470nm）激发，且转移其能量到受体荧光团Cy5.5。受体荧光团在约705nm发射。这些荧光团和猝灭剂仅是示范性的。能够经受FRET的任何荧光团，及可以猝灭荧光的任何猝灭剂适合于本发明使用。生产基于FRET探针的方法，在2009年出版的Jothikumar等人的BioTechniques，第46(7):519-524页中进行了描述。

[0184] 在一些实施例中，单一的杂交探针可用于每个分析物。为了利用多颜色编码（例如，如表3中所示），每个探针可以以多个荧光团在预确定的比例标记。比例可被确定，在相同的颜色中，使得阳性对照信号与相同的强度的其它阳性对照信号提供相同的强度。这种方法减少了必须被合成的探针的数目，且可能比利用多个探针的方法更便宜地配置。此外，在杂交探针的情况下，相比多个探针，一个杂交探针配合分析物的序列更容易。

[0185] 虽然本发明的许多方面使用基于核酸的探针被例举，但是本领域的一个普通技术人员将容易地认识到，其它形式的探针将与在本公开中描述的本发明同样一起工作得很好。例如，对分析物特异的结合分子可用作探针。非限制性的示例性的结合分子包括识别

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的分析物，并在分析物存在时产生信号的抗体。

[0186] 在利用荧光标记的本发明的实施方案中，可以使用任何合适的荧光标记。适用于本发明使用的示例性的荧光标记包括罗丹明、对甲氨基酚、荧光素、硫代荧光素(thiofluorescein)、氨基荧光素、羧基荧光素、氯代荧光素、甲基荧光素、磺基荧光素、氨基对甲氨基酚(aminorhodol)、羧基对甲氨基酚(carboxyrhodol)、氯代对甲氨基酚(chlororhodol)、甲基对甲氨基酚(methylrhodol)、磺基对甲氨基酚(sulforhodol)、氨基罗丹明(aminorhodamine)、羧基罗丹明、氯代罗丹明(chlororhodamine)、甲基罗丹明(methylrhodamine)、磺基罗丹明(sulforhodamine)、及硫代罗丹明(thiorhodamine)、青色素(cyanine)、吲哚碳青色素、氧碳青色素(oxacarbocyanine)、噻碳青色素(thiacarbocyanine)、部青色素(merocyanine)、青色素2、青色素3、青色素3.5、青色素5、青色素5.5、青色素7、恶二唑衍生物(oxadiazole derivatives)、吡啶恶唑(pyridyloxazole)、硝基苯并恶二唑(nitrobenzoxadiazole)、苯并恶二唑(benzoxadiazole)、芘衍生物(pyrene derivatives)、级联蓝、恶嗪衍生物、尼罗红、尼罗蓝、甲酚紫、恶嗪170、吖啶衍生物、二氨基吖啶(proflavin)、吖啶橙、吖啶黄、金胺(arylmethine)衍生物、金胺、结晶紫、孔雀石绿、四吡咯衍生物、卟吩、酞青色素和胆红素。示例性的猝灭剂包括黑洞猝灭剂，如BHQ-0、BHQ-1、BHQ-2、BHQ-3；ATTO猝灭剂，如ATTO 540Q、ATTO 580Q和ATTO 612Q；等等。

[0187] 可以与本发明使用的荧光团不限于本文描述的任何荧光团。例如，具有改进性能的荧光团不断发展，且这些荧光团可以很容易地与本公开提供的方法使用。这种改进的荧光团包括量子点，在单一波长被激发后，其可以以不同的波长发射能量。使用这样的荧光团的优点在于，仅需要一个单一的激发源，但是许多不同的信号可以被定量，例如，根据颜色和强度被定量。此外，具有窄的发射光谱的荧光团将对本文描述的方法特别有用，因为这样的荧光团可被包括在其发光光谱之间很少重叠或没有重叠的多路测定中，从而提供更多的“颜色”并显著地提高编码的分析物的总体数目。

[0188] 在一些情况下，一种或更多种试剂被冻干。任何合适的试剂可以被冻干。例如，探针、引物、酶、抗体或用于检测的任何其它试剂可被冻干。在一些情况下，包括分析物的样品也可被冻干。冻干可能是有用的，例如，当在发展中地区分配试剂和/或样品时，在这些地区获得冷库是昂贵的、不易利用的、或不能可靠地利用的。在一个实例中，冻干的试剂包括在本公开中描述的或以其它方式适合于本发明使用的任何探针或分析物特异性试剂。在另一个实例中，冻干的试剂包括PCR引物。在又另一实例中，冻干的试剂包括适合用于执行PCR反应的试剂。

[0189] V. 分析技术与仪器

[0190] 本文提供的方法适合与各种检测方法使用。例如，可应用的方法使用测量荧光信号的波长和强度的分析技术。这可以通过测量整个光谱波长的信号的强度实现，或通过使用限制某些波长的光的带通滤波器，从而仅允许某些波长的光到达光电检测器实现。许多实时PCR和定量PCR仪器包括激发光源和带通滤波器，其能够检测在四种颜色(例如，蓝色、绿色、黄色和红色)上的荧光信号。因此，本发明的方法可以使用本领域广泛使用的仪器容易地应用。重要的是，本文提供的方法和组合物可用于通过获得单一溶液的累积测量结果来检测多种分析物。没有分离的必要。本发明不需要使用珠或固相。当然，本领域的

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一个普通技术人员应当理解，如果需要的话，本发明可以结合分离、珠、或固相使用。

[0191] 图 5 显示了可用于获得对本发明的方法有用的累积测量结果的仪器的配置的两个实例。参考图 5，带有配置为检测波长和强度两者的检测器（例如，荧光计）的仪器示于 501。参考 501，至少一个激发光源 502 被引导到含有分析物和在分析物 504 存在时产生信号的试剂的室 503 中。如在其他地方描述的，分析物可以是核酸，且产生信号的试剂可以是包括荧光团和猝灭剂的杂交探针。如果分析物存在，则产生发射信号 505。在 501 中所示的配置中，该发射信号的波长和强度通过能够产生荧光发射光谱 506 的检测器在整个光谱上测量。

[0192] 波长和强度也可以使用光电检测器和带通滤波器的组合确定。该配置在本领域中已知的几种热循环仪中使用。参考图 5，具有带通滤波器和光点检测器的仪器在 507 中描绘。参考 507，至少一个激发光源 508 被引导到含有分析物和在分析物 510 存在时产生信号的试剂的室 509 中。如在其他地方描述的，分析物可以是核酸，且产生信号的试剂可以是包括荧光团和猝灭剂的杂交探针。如果分析物存在，则产生发射信号 511。在 507 所示的配置中，带通滤波器 512、513 和 514 被用于限制光的通过，以在特定的波长范围内发光。例如带通滤波器 512 可以限制光的通过，以在波长范围 1 内发光。带通滤波器 513 可以限制光的通过，以在波长范围 2 内发光。带通滤波器 514 可以限制光的通过，以在波长范围 3 内发光。任何数目的带通滤波器（例如，1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、或更多）可用于本发明。此外，在 507 中带通滤波器的位置不意味着是限制性的。在一些情况下，发射的光可在任何时间通过多于一个的带通滤波器。在其它情况下，带通滤波器可以被配置为使得所有发射的光在一个时间仅通过一个带通滤波器。通过带通滤波器后，过滤的光 515、516 和 517 通过光电检测器检测。例如，过滤的光 515 可能是在波长范围 1 内的光，过滤的光 516 可能是在波长范围 2 内的光，且过滤的光 517 可能是在波长范围 3 内的光。然后每个过滤的光 515、516、及 517 的强度可以通过光电检测器 518 定量。

[0193] 本公开中描述的方法可与各种扩增方法兼容，包括定量 PCR(qPCR) 法、终点 PCR 法、逆转录酶 PCR、及数字 PCR 法。数字 PCR 法（例如，微滴数字 (BIORAD) 和动态阵列 (FLUIDIGM)）产生高度敏感的多核苷酸拷贝数目的定量。本文提供的方法可以容易地集成到这些系统中，通过允许在微滴或动态阵列中的多路显著地扩大其工作能力。

[0194] 类似地，本公开中描述的方法可在实时 PCR 测定中应用。例如，随着 PCR 运行完成，实时数据可以被完全记录。然后端点值可被解码以指示分析物的存在或不存在。通过检测该颜色的实时曲线的二阶导数的最大值，可对每个颜色分析各个循环阈值 (Ct) 的值。因为特定的分析物被扩增，荧光团以与该序列编码的颜色多样性相同的比例从其相应的探针释放。因此，如 1100 代码在蓝色和绿色中将具有相同的 Ct，而 1400 代码将具有领先其蓝色 Ct2 个循环的绿色 Ct。这种额外的数据能够测定每个分析物的标识和起始量。

[0195] 本文描述的方法也可以直接在组织样品上使用。例如，可以获得组织样品，且使用光刻法在组织样品上直接建造孔 (well)。组织样品可以被事先固定以建造孔。然后可通过分配适当的试剂进入组织样品中的孔中以分析组织样品。本公开中描述的编码和解码方法可用于蚀刻到组织中的孔中的分析物的多路检测。每个孔可以对应于单一细胞或若干细胞，当对组织的不同部分执行分析物分析时，提供优异的空间分辨率。这个方法可以被用于

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检测相同组织的不同区域的分析物。

[0196] 在一些情况下，仪器可被修改或构建，例如，以提供在多波长的额外的激发光源，和 / 或提供额外的带通滤波器或确定完整的光谱的能力。包括额外的激发源将允许更大数量的各种荧光团的激发。包括额外的带通滤波器，或者修改或构建仪器能够确定整个光谱，允许检测来自荧光团的更多种发射。这些技术可用于增加可以用于本公开中描述的方法的荧光团的数目，且因此可以增加被同时检测的分析物的数目。

[0197] 在一些情况下，本公开中描述的方法利用终点 PCR 法。然而，在一些情况下，结果可在终点前获得。例如，当尽快需要结果时，这可能是有利的。例如，在一种执行校准实验的情况下，其中分析分析物的不同的起始浓度，以确定信号达到饱和其循环数，其为分析物的起始量的函数。使用这种数据，实时监控 PCR 反应的计算机，可被编程以根据特定系统的检测极限 (LOD) 的限制搜索达到最大的循环数的饱和。如果到最大循环数时，分析物没有扩增 (例如，除了可能存在的阳性对照)，那么对于该分析物的结果是阴性。如果到最大循环数时，分析物有扩增，那么对于该分析物的结果是阳性，且在每个颜色中的饱和强度可以被用于使用编码方案解码结果。在这两种情况下，没有必要运行 PCR 反应超过由 LOD 为仪器设定的循环的数目。尽管 PCR 方法已被用于说明这种定量方法，本领域的一个普通技术人员将容易认识到，类似的原理可以在任何催化体系中应用，其中分析物的起始量限制了反应速率，且反应速率可以通过在一段时间内信号强度的演变被测量和报告。

[0198] VI. 组合物和试剂盒

[0199] 本公开还提供了与本文描述的方法使用的组合物和试剂盒。组合物可以包括本文描述的任何成分、反应混合物和 / 或中间体，以及任意组合。例如，本公开提供了与本文提供的方法一起使用的检测试剂。可提供任何合适的检测试剂，包括如在本说明书中其他地方描述的，以荧光团和猝灭剂和引物标记的杂交探针。

[0200] 在一些情况下，组合物包括使用四个荧光团检测至少七个分析物的试剂。在一些情况下，组合物包括使用四个荧光团检测至少 5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或 50 个分析物的试剂。在一些情况下，组合物包括使用五个荧光团检测至少 6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或 50 个分析物的试剂。在一些情况下，组合物包括使用六个荧光团检测至少 7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或 50 个分析物的试剂。在一些情况下，组合物包括使用七个荧光团检测至少 8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或 50 个分析物的试剂。

[0201] 在一些情况下，组合物包括引物。组合物可包括至少 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或 50 个引物配对。

[0202] 本发明还提供了用于实施本发明的方法的试剂盒。因此，各种试剂盒在合适的包装中提供。试剂盒可用于本文描述的任何一个或更多个用途，且，相应地，可含有用于检测多个分析物中的每个分析物的存在或不存在的指令。试剂盒可包括编码方案、或解码矩阵，

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以帮助用户转换累积测量结果为表明多个分析物中的每个分析物的存在或不存在的结果。试剂盒可以是诊断试剂盒，例如，适合用于包括本文列举的分析物的任何分析物的检测的诊断试剂盒。试剂盒可以包含任何本公开中提供的组合物，包括上文列举的那些。

[0203] 图6显示了本发明的示例性试剂盒的示意图。参考图6，显示了包括盒体601的试剂盒，盒体601含有用于分析物（例如，包括荧光团和猝灭剂的多核苷酸探针）的检测的试剂602，及用于核酸的扩增的引物603。试剂盒也可以包含任何其他试剂，例如适合于执行扩增反应的试剂604。在601中描绘的实施方案中，用于分析物的检测的试剂及引物各自包含在单一体积（如管或瓶）中。然而，这些试剂也可以以单独的体积提供，如605中所示的盒体所描绘的。盒体605包含七个单独的体积。三个体积（606、607和608）含有用于分析物的检测的试剂（或这类试剂的混合物），且三个体积（609、610和611）含有引物（或引物的混合物）。试剂盒还可以含有适合于执行扩增反应的试剂604。

[0204] 提供图6仅用于说明的目的。用于分析物（例如，探针）的检测的任何数目的试剂，及任何数目的引物可以被包括在单一的管或瓶中，使其适合应用。例如，在一些实施方案中，在单一的瓶中可以包括1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个试剂，用于分析物的检测。在其它情况下，在单一的瓶或管中可以包括1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个引物，用于扩增。在一些情况下，引物和探针可在相同的瓶或管中提供。例如，每对引物配对可以提供1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个探针。在其它情况下，每对探针配对可以提供1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个引物。试剂盒可以包含1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个瓶或管。对照分析物，及其相关的检测试剂和引物，也可以包括在试剂盒中。这些可以包括在单独的瓶或管中，或者例如，包括在提供用于扩增反应的试剂的瓶或管604中。如在本说明书中其他地方描述的，可以冻干试剂盒中的任何试剂。

[0205] VII. 系统和软件

[0206] 本公开的方法还可以作为系统的一部分执行。例如，可以通过包含在计算机可读介质中的软件执行本文描述的方法的一个或更多个步骤。软件可用于编码分析物、检查简并性、减少或消除简并性、和/或产生解码矩阵。软件也可以用于将仪器（例如，PCR器）上作出的累积测量结果转换为关于分析物的存在或不存在的信息。例如，参考图4，计算机401可以连接到输入装置403、显示器402和打印机404。计算机可以执行本公开中描述的方法的任意一个方法的一个或更多个步骤，例如包括，编码方法410、分析方法420和解码方法430。计算机可减少或消除简并性，例如，作为编码方法410的一部分。类似地，计算机可以产生通过设计是非简并的编码方案，例如使用数学迭代设计的编码方案。计算机也可以连接到网络405。网络可以是局域网和/或因特网。网络可以用于在不同位置的多个计

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算机上执行本发明的方法。例如，一台计算机可以执行编码，另一台计算机可执行测量，且又一台计算机可执行解码。一台计算机可执行这些方法的任何方法或全部。网络可用于在全世界传输使用本公开中描述的方法所获得的信息，例如跨越国界。

[0207] 在一个实例中，软件被嵌入在执行测量的仪器中，例如热循环仪。在另一个实例中，软件被安装在连接到执行测量的仪器的计算机上，例如连接到热循环仪的计算机。在另一个实例中，软件是“云”，即在另一台计算机可通过网络与其通信的计算机上。

[0208] 上文描述的每个元件可被连接到控制器，所述控制器与每个元件通信，并协调它们的操作。例如，控制器可启动将一系列的分析物编码为颜色和强度的值。如本公开全文所描述的，控制器可以执行软件，以通过从编码矩阵中除去一个或更多个潜在的分析物代码来减少或消除简并性。然后，控制器可以自动地从探针的制造商订购对应该代码的探针。可替代地，控制器可以操作可以合成相应的探针的寡核苷酸合成仪。分析了在仪器上的样品（其也可以是自动的）之后，控制器可以使用解码矩阵处理分析的结果。然后，控制器可将这些结果提供给用户。

[0209] VIII. 服务

[0210] 本文提供的方法也可以作为服务执行。例如，服务提供者可以获得客户希望分析的多个分析物的标识。然后服务提供者可以通过本文所述的任何方法编码每个待检测的分析物，并向客户提供适当的试剂用于测定。客户也可以执行测定，并向服务提供者提供结果以解码。然后服务提供者可以向客户提供解码的结果。客户还可以通过与安装在本地（在客户所在地）或远程（例如，在通过网络可达的一台服务器上）的软件交互，;来编码分析物、产生探针、和 / 或解码结果。示例性的客户包括临床实验室、医生、食品和消费品制造商、工业制造商（例如，石油公司）等等。客户或团体可以是有需要或希望使用本发明的方法、系统、组合物和试剂盒的任何合适的客户或团体。

实施例

[0211] 实施例 1 :一般材料和方法

[0212] 1. 一般试剂

[0213] TE 缓冲液，pH7(LIFE TECHNOLOGIES,Carlsbad,CA) ; 无 RNA 酶超纯水 (LIFE TECHNOLOGIES,Carlsbad,CA) ;Taq 5x 预混液 (FISHER SCIENTIFIC COMPANY,Tustin,CA)。

[0214] 2.DNA 序列、引物和探针

[0215] 选择了来自临床相关的生物体的五种核酸用于示例性的研究 :(1) 人类免疫缺陷病毒 1(HIV-1) ;(2) 恶性疟原虫（疟疾）;(3) 单纯性疱疹病毒 2(HSV-2) ;(4) 结核分枝杆菌 (TB) ;和 (5) 登革热病毒 3 型（登革热）。

[0216] 来自 HIV-1 的基因组的诊断相关的两个区域，p17 和聚蛋白酶，分别选自 Los Alamos 国家实验室的 HIV-1 参考序列。来自恶性疟原虫 ChR 7 基因的诊断序列从 UCSC 恶性疟原虫基因组浏览器获得。单纯性疱疹病毒 2 序列从欧洲分子生物学库 (European Molecular Biology Library) 中获得的序列合成。相似地，结核分歧杆菌中的 rpoB 基因的诊断序列合成自从欧洲分子生物学库获得的序列。登革热病毒 3 型的 PCR 诊断序列获得自美国国家卫生研究院 (National Institute of Health) 的健康基因序列数据库。

[0217] 由 INTEGRATED DNA TECHNOLOGIES(IDT) 合成寡核苷酸。使用来自 IDT 的 OLIGO 分

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析仪选择用于每个分析物的探针和引物配对。对所有分析物合成包含在 5’ 端的荧光团和在 3’ 端的猝灭剂的感测探针。冻干所有的寡核苷酸，并在使用前在 TE 缓冲液中重建。表 9-14 显示了六个分析物的每个分析物的序列信息、目标序列、引物、和探针。

[0218] 表 9. HIV-1 聚蛋白酶的序列信息。

[0219]

序列信息	从碱基 2253-2550 合成的 HIV-1 多蛋白酶 198mer
来源	HIV-1 参考序列，Los Alamos 国家实验室
目标序列（3’至 5’）	GGAAGCTCTATTAGATACAGGAGCAGATGAT ACAGTATTAGAAGAAATGA

[0220]

	GTTTGCCAGGAAGATGGAAACCAAAAATGAT AGGGGGAATTGGAGGTTTT ATCAAAGTAAGACAGTATGATCAGATACTCA TAGAAATCTGTGGACATAA AGCTATAGGTACAGTATTAGTAGGACCTACA CCTGTCAACATAATTGG
正向引物（3’至 5’）	GGAAGCTCTATTAGATACAGGAGCAG
反向引物（3’至 5’）	CCAATTATGTTGACAGGTGTAGGTCC
探针 1（3’至 5’）	**/56-FAM**/TGAGTTTGCCAGGAAGATGGAAAC CA/3BHQ_1/

[0221] 表 10. HIV p17 的序列信息。

[0222]

序列信息	从碱基 790-1186 合成的 HIV-1 P17 199mer
来源	HIV-1 参考序列，Los Alamos 国家实验室
目标序列（3’至 5’）	CAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAG ATCATTATA TAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGA GATAAAAG ACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAA CAAAAGTAAG AAAAAAGCACAGCAAGCAGCAGCTGACACAGGACACAGC AATCAGGTCA
正向引物（3’至 5’）	CAGCTACAACCATCCCTTCAGACA
反向引物（3’至 5’）	TGACCTGATTGCTGTGTCCTGTGT
探针 1（3’至 5’）	**/56-FAM**/AGCAACCCTCTATTGTGTGCATCAAAGG/3BHQ_1
探针 2（3’至 5’）	**/5Cy3**/AAAGCACAGCAAGCAGCAGCTGA/3BHQ_2/

[0223] 表 11. 疟原虫 ChR 7 的序列信息。

[0224]

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序列信息	从碱基 1139138-1141223 合成的疟原虫 ChR7 199mer
来源	UCSC 恶性疟原虫基因组浏览器网关
目标序列（3’至 5’）	GCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCG ATTCCGGAGAGGGAGCCTGAGAAATAGCTACCACATCTAAG GAAGGCAGCAGGCGCGTAAATTACCCAATTCTAAAGAAGAG AGGTAGTGACAAGAAATAACAATGCAAGGCCAATTTAAAAC CTTCCCAGAGTAACAATTGGAGGGCAAGTCTGGTG
正向引物（3’至 5’）	GCCTAACATGGCTATGACGGGTAA
反向引物（3’至 5’）	CACCAGACTTGCCCTCCAATTGTT
探针 1（3’至 5’）	/56-FAM/ATTCCGGAGAGGGAGCCTGAGAAATA/3BHQ_1/
探针 2（3’至 5’）	/56-ROXN/AAGGAAGGCAGCAGGCGCGTAAATTA/3BHQ_2/

[0225] 表 12. 单纯性疱疹病毒 2 的序列信息。

[0226]

序列信息	从 HSV-2 基因组合成的 HSV-2 193mer
来源	EMBL 库 AJ303204
目标序列（3’至 5’）	TCAGCCCATCCTCCTTCGGCAGTATGGAGGGTGTCGCGGCG GCGAGCCGCCGTCCCCAAAGACGTGCGGGTCGTACACGTA CACGTACCAGGGCGGCGGGCCTCCGACCCGGTACGCTCTCG TAAATGCTTCCCTGCTGGTGCCGATCTGGGACCGCGCCGCG GAGACATTCGAGTACCAGATCGAACTCGG
正向引物（3’至 5’）	TCAGCCCATCCTCCTTCGGCAGTAT
反向引物（3’至 5’）	CCGAGTTCGATCTGGTACTCGAATGT
探针 1（3’至 5’）	/56-FAM/AAAGACGTGCGGGTCGTACACGTACA/3BHQ_1/
探针 2（3’至 5’）	/5Cy5/TAAATGCTTCCCTGCTGGTGCCGAT/3IAbRQSp/

[0227] 表 13. 结核分枝杆菌 rpoB 的序列信息。

[0228]

序列信息	从结核分枝杆菌 rpoB 基因组合成的结核分枝杆菌 rpoB 200mer
来源	EMBL 库 GQ395623
目标序列（3’至 5’）	GAGTGCAAAGACAAGGACATGACGTACGCGGCCCCGCTGTT CGTCACGGCCGAGTTCATCAACAACAACACCGGCGAGATCA AGAGCCAGACGGTGTTCATGGGTGACTTCCCGATGATGACCG AGAAGGGCACCTTCATCATCAACGGCACCGAGCGCGTCGTG GTCAGCCAGCTGGTCCGCTCGCCCGGTGTGTACTT
正向引物（3’至 5’）	GAGTGCAAAGACAAGGACATGACG
反向引物（3’至 5’）	AAGTACACACCGGGCGAGC
探针 1（3’至 5’）	/56-FAM/CGCTGTTCGTCACGGCCGAGTTCAT/3BHQ_1/
探针 2（3’至 5’）	/5Cy3/AGATCAAGAGCCAGACGGTGTTCATG/3BHQ_2/
探针 3（3’至 5’）	/5Cy5/AAGGGCACCTTCATCATCAACGGCA/3IAbRQSp/

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[0229] 表 14. 登革热病毒 3 型的序列信息。

[0230]

序列信息	从登革热病毒 3 型基因组合成的登革热 3 型 200mer
来源	GenBank M93130
目标序列（3’至 5’）	ATGCCAACTGTGATTGAGCACTTAGAAAGACTACAAAGGAA ACATGGAGGAATGCTTGTGAGAAATCCACTCTCACGAAACTC CACGCACGAAATGTATTGGATATCCAATGGTACAGGCAACATC GTCTCATTCACAATGACACACAGGAGACCCACCATAGAGAAA GATGTGGATTTAGGAGCAGGAACCCGACATGT
正向引物（3’至	ATGCCAACTGTGATTGAGCACT

[0231]

5’）	
反向引物（3’至 5’）	ACATGTCGGGTTCCTGCTCCTAAA
探针 1（3’至 5’）	/56-FAM/ACAAAGGAAACATGGAGGAATGCTTGTGA/3BHQ_1 /
探针 2（3’至 5’）	/5Cy3/ACTCTCACGAAACTCCACGCACGAAA/3BHQ_2/
探针 3（3’至 5’）	/56-ROXN/ACAATGACACACAGGAGACCCACCAT/3BHQ_2/
探针 4（3’至 5’）	/5Cy5/GGATATCCAATGGTACAGGCAACATCGT/3IAbRQSp/

[0232] 3. 聚合酶链式反应

[0233] 所有的 PCR 反应在 ROCHE 480LIGHTCYCLER 仪器上执行。以在 95℃，60 秒的热启动，执行 45 个循环。变性、退火（annealing）和延伸的循环条件为分别在 95℃、65℃和 70℃分别为 45 秒、50 秒和 60 秒。每个实验以 15μL 的反应体积一式五份运行。每个退火步骤后，在以下通道中获得荧光测量结果：483nm 至 533nm（FAM）、523nm 至 568nm（Cy3）、558nm 至 610nm（ROX）、及 615nm 至 670nm（Cy5）。在热启动和 45 个热循环结束后也可以获得荧光测量结果。这两个测量结果之间的荧光强度的变化（热启动后，及在 45 个循环结束时）确定终点荧光信号。

[0234] 实施例 2：基于每个分析物使用多于一个颜色的编码方法同时检测来自病原体的多核苷酸标记

[0235] 本实施例描述了选自 HIV（2 个多核苷酸）、HSV-2、分枝杆菌、疟原虫、和登革病毒的最多四种多核苷酸的同时检测。使用表 15 中描述的试剂，装配（assemble）了 14 个 PCR 反应。

[0236] 表 15. 14 个 PCR 反应的试剂。

[0237]

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反应数	试剂类型	试剂	浓度	体积
1		超纯水	-	34 μL
		Taq 5x 预混液	-	42 μL
	模板	聚蛋白酶模板	17 nM	2 μL
	引物	聚蛋白酶 FWD 引物	1 nM	2 μL
		蛋白酶 RVS 引物	1 nM	2 μL
	探针	聚蛋白酶探针 1	1 nM	2 μL
2		超纯水	-	28 μL
		Taq 5x 预混液	-	42 μL
	模板	疟疾模板	15 nM	2 μL

[0238]

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		疱疹模板	17 nM	2 μL
		TB 模板	16 nM	2 μL
		登革热病毒模板	17 nM	2 μL
	引物	聚蛋白酶 FWD 引物	1 nM	2 μL
		蛋白酶 RVS 引物	1 nM	2 μL
	探针	聚蛋白酶探针 1	1 nM	2 μL
3		超纯水	-	32 μL
		Taq 5x 预混液	-	42 μL
	模板	疟疾模板	15 nM	2 μL
	引物	疟疾 FWD 引物	1 μM	2 μL
		疟疾 RVS 引物	1 μM	2 μL
	探针	疟疾探针 1	1 μM	2 μL
		疟疾探针 2	1 μM	2 μL
4		超纯水	-	26 μL
		Taq 5x 预混液	-	42 μL
	模板	聚蛋白酶模板	17 nM	2 μL
		疱疹模板	17 nM	2 μL
		TB 模板	16 nM	2 μL
		登革热病毒模板	17 nM	2 μL
	引物	疟疾 FWD 引物	1 μM	2 μL
		疟疾 RVS 引物	1 μM	2 μL
	探针	疟疾探针 1	1 μM	2 μL
		疟疾探针 2	1 μM	2 μL
5		超纯水	-	32 μL
		Taq 5x 预混液	-	42 μL
	模板	疱疹模板	17 nM	2 μL
	引物	疱疹 FWD 引物	1 μM	2 μL
		疱疹 RVS 引物	1 μM	2 μL
	探针	疱疹探针 1	1 μM	2 μL
		疱疹探针 2	1 μM	2 μL
6		超纯水	-	26 μL
		Taq 5x 预混液	-	42 μL
	模板	聚蛋白酶模板	17 nM	2 μL
		疟疾模板	15 nM	2 μL
		TB 模板	16 nM	2 μL
		登革热病毒模板	17 nM	2 μL
	引物	疱疹 FWD 引物	1 μM	2 μL
		疱疹 RVS 引物	1 μM	2 μL
	探针	疱疹探针 1	1 μM	2 μL
		疱疹探针 2	1 μM	2 μL
7		超纯水	-	30 μL
		Taq 5x 预混液	-	42 μL
	模板	TB 模板	16 nM	2 μL

[0239]

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	引物	TB FWD 引物	1 μM	2 μL
		TB RVS 引物	1 μM	2 μL
	探针	TB 探针 1	1 μM	2 μL
		TB 探针 2	1 μM	2 μL
		TB 探针 3	1 μM	2 μL
8		超纯水	-	30 μL
		Taq 5x 预混液	-	42 μL
	模板	聚蛋白酶模板	17 nM	2 μL
		疟疾模板	15 nM	2 μL
		疱疹模板	17 nM	2 μL
		登革热病毒模板	17 nM	2 μL
	引物	TB FWD 引物	1 μM	2 μL
		TB RVS 引物	1 μM	2 μL
	探针	TB 探针 1	1 μM	2 μL
		TB 探针 2	1 μM	2 μL
		TB 探针 3	1 μM	2 μL
9		超纯水	-	28 μL
		Taq 5x 预混液	-	42 μL
	模板	登革热病毒模板	17 nM	2 μL
	引物	登革热病毒 FWD 引物	1 μM	2 μL
		登革热病毒 RVS 引物	1 μM	2 μL
	探针	登革热病毒探针 1	1 μM	2 μL
		登革热病毒探针 2	1 μM	2 μL
		登革热病毒探针 3	1 μM	2 μL
		登革热病毒探针 4	1 μM	2 μL
10		超纯水	-	22 μL
		Taq 5x 预混液	-	42 μL
	模板	聚蛋白酶模板	17 nM	2 μL
		疟疾模板	15 nM	2 μL
		疱疹模板	17 nM	2 μL
		TB 模板	16 nM	2 μL
	引物	登革热病毒 FWD 引物	1 μM	2 μL
		登革热病毒 RVS 引物	1 μM	2 μL
	探针	登革热病毒探针 1	1 μM	2 μL
		登革热病毒探针 2	1 μM	2 μL
		登革热病毒探针 3	1 μM	2 μL
		登革热病毒探针 4	1 μM	2 μL
11		Taq 5x 预混液	-	42 μL
	模板	聚蛋白酶模板	17 nM	2 μL
		疟疾模板	15 nM	2 μL
		疱疹模板	17 nM	2 μL
		登革热病毒模板	17 nM	2 μL
	引物	聚蛋白酶 FWD 引物	1 μM	2 μL

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		聚蛋白酶 RVS 引物	1 μM	2 μL
		疟疾 FWD 引物	1 μM	2 μL
		疟疾 RVS 引物	1 μM	2 μL
		疱疹 FWD 引物	1 μM	2 μL
		疱疹 RVS 引物	1 μM	2 μL
		登革热病毒 FWD 引物	1 μM	2 μL
		登革热病毒 RVS 引物	1 μM	2 μL
	探针	聚蛋白酶探针 1	1 μM	2 μL
		疟疾探针 1	1 μM	2 μL
		疟疾探针 2	1 μM	2 μL
		疱疹探针 1	1 μM	2 μL
		疱疹探针 2	1 μM	2 μL
		登革热病毒探针 1	1 μM	2 μL
		登革热病毒探针 2	1 μM	2 μL
		登革热病毒探针 3	1 μM	2 μL
		登革热病毒探针 4	1 μM	2 μL
12		超纯水	-	2 μL
		Taq 5x 预混液	-	42 μL
	模板	聚蛋白酶模板	17 nM	2 μL
		疟疾模板	15 nM	2 μL
		疱疹模板	17 nM	2 μL
		TB 模板	16 nM	2 μL
	引物	聚蛋白酶 FWD 引物	1 μM	2 μL
		聚蛋白酶 FWD 引物	1 μM	2 μL
		疟疾 FWD 引物	1 μM	2 μL
		疟疾 RVS 引物	1 μM	2 μL
		疱疹 FWD 引物	1 μM	2 μL
		疱疹 RVS 引物	1 μM	2 μL
		TB FWD 引物	1 μM	2 μL
		TB RVS 引物	1 μM	2 μL
	探针	聚蛋白酶探针 1	1 μM	2 μL
		疟疾探针 1	1 μM	2 μL
		疟疾探针 2	1 μM	2 μL
		疱疹探针 1	1 μM	2 μL
		疱疹探针 2	1 μM	2 μL
		TB 探针 1	1 μM	2 μL
		TB 探针 2	1 μM	2 μL
		TB 探针 3	1 μM	2 μL
13		超纯水	-	12 μL
		Taq 5x 预混液	-	42 μL
	模板	聚蛋白酶模板	17 nM	2 μL
		疱疹模板	17 nM	2 μL
		TB 模板	16 nM	2 μL

[0241]

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	引物	聚蛋白酶 FWD 引物	1 μM	2 μL
		聚蛋白酶 FWD 引物	1 μM	2 μL
		疱疹 FWD 引物	1 μM	2 μL
		疱疹 RVS 引物	1 μM	2 μL
		TB FWD 引物	1 μM	2 μL
		TB RVS 引物	1 μM	2 μL
	探针	聚蛋白酶探针 1	1 μM	2 μL
		疱疹探针 1	1 μM	2 μL
		疱疹探针 2	1 μM	2 μL
		TB 探针 1	1 μM	2 μL
		TB 探针 2	1 μM	2 μL
		TB 探针 3	1 μM	2 μL
14		超纯水	-	10 μL
		Taq 5x 预混液	-	42 μL
	模板	聚蛋白酶模板	17 nM	2 μL
		疟疾模板	15 nM	2 μL
		登革热病毒模板	17 nM	2 μL
	引物	聚蛋白酶 FWD 引物	1 μM	2 μL
		聚蛋白酶 FWD 引物	1 μM	2 μL
		疟疾 FWD 引物	1 μM	2 μL
		疟疾 RVS 引物	1 μM	2 μL
		登革热病毒 FWD 引物	1 μM	2 μL
		登革热病毒 RVS 引物	1 μM	2 μL
	探针	聚蛋白酶探针 1	1 μM	2 μL
		疟疾探针 1	1 μM	2 μL
		疟疾探针 2	1 μM	2 μL
		登革热病毒探针 1	1 μM	2 μL
		登革热病毒探针 2	1 μM	2 μL
		登革热病毒探针 3	1 μM	2 μL
		登革热病毒探针 4	1 μM	2 μL

[0242] 参照表 15，在其它分析物不存在时，反应 1、3、5、7 和 9（分别包含 HIV 聚蛋白酶、疟原虫、HSV、分枝杆菌和登革热的分析物）是为每个多核苷酸分析物单独提供基线荧光强度的阳性对照。如下文更充分地描述的，在每个颜色中荧光强度的变化被用于提供色谱（图 3）中每个颜色的预期累积的强度水平。反应 2、4、6、8 和 10 是阴性对照，分别用于确定非特异性扩增的程度。在这些反应中，提供的引物对提供的分析物特不是异性的。

[0243] 实验 11、12、13 和 14 是多路检测实验。如表 16 中表示的执行编码。在每个颜色中，对每个测定测量的荧光强度被描绘为图 3 的色谱中的黑色圆圈。

[0244] 表 16. 病原体检测的编码方案。

[0245]

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	FAM	Cy3	ROX	Cy5
HIV 聚蛋白酶	1	0	0	0
HIV p17	1	1	0	0
疟原虫	1	0	1	0
HSV	1	0	0	1
分歧杆菌	1	1	0	1
登革热病毒	1	1	1	1

[0246] 为了构建色谱，在每个颜色中，阳性对照的信号（反应 1、3、5、7 和 9）被用于装配存在的序列的每个可能的组合。累积信号被绘制在称为“色谱”（图 3）的图中。对应于相同颜色中相同秩的所有可能组合的累积信号被组织到其自己的“带”内。对所有四个颜色这样做产生水平（level）和带结构，针对其可以评估每个组合的实验结果（反应 11、12、13 和 14）。图 3 显示了色谱的三个重复，所述色谱使用阳性对照样品构建，并以实验结果 4112、3121 和 3102 叠加在色谱上。实验结果由黑点指示。

[0247] 在反应 11-14 中每个颜色的强度值以任意荧光单位（AFU）测量，绘制在色谱中，且然后基于两个标准分配至带。首先，如果实验结果在带内，那么结果被分配到多路性的带（例如，在图 3 的最左边的色谱中为 1 的 Cy3 值）。第二，如果实验结果在两个带之间，则使用两步分析。如果实验结果在两个合理的结果之间，那么实验结果被分配到最接近的带（例如，在图 3 的最右边的色谱中 Cy5 的值 2）。如果实验结果在合理的结果和不合理的结果之间，结果被分配至带的合理的结果（例如，图 3 的中间的色谱中 FAM 的值 3）。图 8 示意性地显示了 4112 的合理的结果（上图），其指示了 HIV 聚蛋白酶、疟原虫、单纯性疱疹、及分枝杆菌的存在；以及 4110 的不合理的结果（下图），其不能被解码并指示了测定中的故障。

[0248] 使用这些标准，实验 12 的输出被指定为 4112，这是正确的答案。实验 14 的输出可以被指定为 2121 或 3121，但 2121 是不合理的，因此其结果必然是 3121，这是正确的答案。实验 13 的输出可以被指定为 3102 或 3101，这两者都是合理的，但在 Cy5 通道中相比 1，结果明确地更接近 2，所以正确答案 3102，被指定为的结果。这些结果提供了本文提供的方法的实验性验证。

[0249] 研究结果表明组合信号比可以预期的来自阳性对照信号的简单总和略低的趋势。这可能是因为信号的组合不是完美的线性。这可能是目前不能解释的色谱构建的额外效应的结果。这些效应可能与猝灭剂的绝对浓度相关。猝灭剂的绝对浓度越高，其猝灭的反应体积的百分比越大，使得释放的相同的百分比的荧光团对累积信号的贡献没有预期的那么多。这个问题一个解决方法是使用低浓度的探针，使得信号中的百分损失是可以忽略的，而不管有多少猝灭剂被释放。

[0250] 图 3 显示了包含多个组合的每个秩具有相对宽的带。带的宽度最终是阳性对照样品的最高和最低累积信号之间的差异。如果在特定颜色中所有阳性对照产生完全相同的荧光信号，则每个带的宽度将是零。因为代替的那些信号有所不同，得到的带是相对宽的。然

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而，有一种简单的手段以收紧带。如在上述实验完成的，与加载相同浓度的所有探针不同，作为代替，每个探针的浓度可被调整，使得每个得到的阳性对照信号与相同的颜色中的其它的是相同的。这种方法会显著收紧带，使其更容易确定含有分析物的样品的信号的多路性。

[0251] 实施例 3：基于每个分析物使用一个颜色和不同强度的编码方法同时检测来自病原体的多核苷酸标记

[0252] 本实施例描述了选自登革病毒、HIV p17 和 HIV 聚蛋白酶的最多三种多核苷酸的同时检测。编码方案如表 11 中描绘的。如表 11 所示，每个分析物被编码为单一颜色中荧光强度的单一值。在这种情况下，如本公开其他地方描述的，编码方案通过设计是非简并的。这通过向每个分析物分配强度值实现，所分配的强度值等于之前的分析物的累积强度值加一。当然，如在本说明书中其他地方描述的，初始分配值可以大于 1，例如，以更好地区别噪声。类似地，分配给每个分析物的值可以大于之前的分析物的累积强度值加一，例如，为了增加强度之间的间隔，并使得强度分配更简单。

[0253] 表 17. 检测登革热病毒、HIV 聚蛋白酶和 HIV p17 的编码方案。

[0254]

	FAM
登革热病毒	1
HIV p17	2
HIV 聚蛋白酶	4

[0255] 实施例 2 中使用的相同的试剂（例如，模板、引物和探针）用于此处。测量了由阳性对照终点 PCR 反应中的各个探针产生的荧光信号，并将其用于计算将产生 1x、2x、和 4x 的信号强度的探针的浓度。然后设置实验用于检测分析物出现的七个非空的组合。表 18-24 提供了这些混合物的每个的分析物 PCR 反应成分。

[0256] 表 18. 二进制 FAM 实验 1 混合液 (Cocktail)：HIV 聚蛋白酶、HIV p17、及登革热病毒。

[0257]

试剂	浓度	添加的体积
超纯水	-	50 μL
Taq 5x 预混液	-	25 μL
模板	浓度	添加的体积
聚蛋白酶模板	17nM	4 μL
P17 模板	15nM	4 μL

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登革热病毒模板	17nM	4μL
引物	浓度	添加的体积
聚蛋白酶 FWD 引物	1μM	4μL
聚蛋白酶 RVS 引物	1μM	4μL
P17 FWD 引物	1μM	4μL
P17 RVS 引物	1μM	4μL
登革热病毒 FWD 引物	1μM	4μL
登革热病毒 RVS 引物	1μM	4μL
探针	浓度	添加的体积
聚蛋白酶探针 1	800nM	4μL
P17 探针 1	400nM	4μL
登革热病毒探针 1	200nM	4μL

[0258] 表 19. 二进制 FAM 实验 2 混合液：登革热病毒和 HIV p17。

[0259]

试剂	浓度	添加的体积
超纯水	-	54μL
Taq 5x 预混液	-	25μL
模板	浓度	添加的体积
聚蛋白酶模板	17nM	-
P17 模板	15nM	4μL
登革热病毒模板	17nM	4μL
引物	浓度	添加的体积
聚蛋白酶 FWD 引物	1μM	4μL
聚蛋白酶 RVS 引物	1μM	4μL

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P17 FWD 引物	1μM	4μL
P17 RVS 引物	1μM	4μL
登革热病毒 FWD 引物	1μM	4μL
登革热病毒 RVS 引物	1μM	4μL
探针	浓度	添加的体积
聚蛋白酶探针 1	800nM	4μL
P17 探针 1	400nM	4μL
登革热病毒探针 1	200nM	4μL

[0260] 表 20. 二进制 FAM 实验 3 混合液 :登革热病毒和 HIV 聚蛋白酶。

[0261]

试剂	浓度	添加的体积
超纯水	-	54μL
Taq 5x 预混液	-	25μL
模板	浓度	添加的体积
聚蛋白酶模板	17nM	4μL
P17 模板	15nM	-
登革热病毒模板	17nM	4μL
引物	浓度	添加的体积
聚蛋白酶 FWD 引物	1μM	4μL
聚蛋白酶 RVS 引物	1μM	4μL
P17 FWD 引物	1μM	4μL
P17 RVS 引物	1μM	4μL
登革热病毒 FWD 引物	1μM	4μL
登革热病毒 RVS 引物	1μM	4μL

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探针	浓度	添加的体积
聚蛋白酶探针 1	800nM	4μL
P17 探针 1	400nM	4μL
登革热病毒探针 1	200nM	4μL

[0262] 表 21. 二进制 FAM 实验 4 混合液 :HIV 聚蛋白酶和 HIV p17。

[0263]

试剂	浓度	添加的体积
超纯水		54μL
Taq 5x 预混液	–	25μL
模板	浓度	添加的体积
聚蛋白酶模板	17nM	4μL
P17 模板	15nM	4μL
登革热病毒模板	17nM	–
引物	浓度	添加的体积
聚蛋白酶 FWD 引物	1μM	4μL
聚蛋白酶 RVS 引物	1μM	4μL
P17 FWD 引物	1μM	4μL
P17 RVS 引物	1μM	4μL
登革热病毒 FWD 引物	1μM	4μL
登革热病毒 RVS 引物	1μM	4μL
探针	浓度	添加的体积
聚蛋白酶探针 1	800nM	4μL
P17 探针 1	400nM	4μL
登革热病毒探针 1	200nM	4μL

[0264] 表 22. 二进制 FAM 实验 5 混合液 :登革热病毒。

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[0265]

试剂	浓度	添加的体积
超纯水	-	58μL
Taq 5x 预混液	-	25μL
模板	浓度	添加的体积
聚蛋白酶模板	17nM	-
P17 模板	15nM	-
登革热病毒模板	17nM	4μL
引物	浓度	添加的体积
聚蛋白酶 FWD 引物	1μM	4μL
聚蛋白酶 RVS 引物	1μM	4μL
P17 FWD 引物	1μM	4μL
P17 RVS 引物	1μM	4μL
登革热病毒 FWD 引物	1μM	4μL
登革热病毒 RVS 引物	1μM	4μL
探针	浓度	添加的体积
聚蛋白酶探针 1	800nM	4μL
P17 探针 1	400nM	4μL
登革热病毒探针 1	200nM	4μL

[0266]

[0267] 表 23. 二进制 FAM 实验 6 混合液 :HIV p17。

[0268]

试剂	浓度	添加的体积
超纯水	-	58μL
Taq 5x 预混液	-	25μL

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模板	浓度	添加的体积
聚蛋白酶模板	17nM	-
P17 模板	15nM	4μL
登革热病毒模板	17nM	-
引物	浓度	添加的体积
聚蛋白酶 FWD 引物	1μM	4μL
聚蛋白酶 RVS 引物	1μM	4μL
P17 FWD 引物	1μM	4μL
P17 RVS 引物	1μM	4μL
登革热病毒 FWD 引物	1μM	4μL
登革热病毒 RVS 引物	1μM	4μL
探针	浓度	添加的体积
聚蛋白酶探针 1	800nM	4μL
P17 探针 1	400nM	4μL
登革热病毒探针 1	200nM	4μL

[0269] 表 24. 二进制 FAM 实验 7 混合液 :HIV 聚蛋白酶。

[0270]

试剂	浓度	添加的体积
超纯水	-	58μL
Taq 5x 预混液	-	25μL
模板	浓度	添加的体积
聚蛋白酶模板	17nM	4μL
P17 模板	15nM	-
登革热病毒模板	17nM	-

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引物	浓度	添加的体积
聚蛋白酶 FWD 引物	1μM	4μL
聚蛋白酶 RVS 引物	1μM	4μL
P17 FWD 引物	1μM	4μL
P17 RVS 引物	1μM	4μL
登革热病毒 FWD 引物	1μM	4μL
登革热病毒 RVS 引物	1μM	4μL
探针	浓度	添加的体积
聚蛋白酶探针 1	800nM	4μL
P17 探针 1	400nM	4μL
登革热病毒探针 1	200nM	4μL

[0271]

[0272] 每个实验的总荧光信号在如上文描述构建的色谱上绘制(图 7)。围绕每个阳性对照测量结果的带的宽度是 1x 测量结果传播的不确定性。该不确定性等于饱和的最后五个 PCR 循环的荧光信号的标准差。

[0273] 图 7 显示了该实验的结果。最右边的三个结果显示了单独的登革病毒 (DEN)、单独的 HIV p17(P17) 和 HIV 聚蛋白酶 (TPP) 的测量强度。对这些分析物的每个分配的强度如表 11 所示,且图 7 确认了三个分析物单独存在时,产生了预期的强度结果(见 DEN、P17 和 TPP)。在测定结束时,基本上所有的探针已被水解,释放了发射信号的基本上所有的荧光团。固定数量的荧光团产生固定的且可预测的信号量。因此,由通过加入到反应体积的探针的数量确定的,存在的目标通常应产生固定量的信号。

[0274] 如预期的,分析物的组合的强度值等于分配的强度之和。因为这种编码方案被设计成非简并的,每个结果可以明确地解码成特定分析物的存在或不存在。例如,从图 7 的左侧开始,7 的结果表明所有三个分析物存在。3 的结果表明登革热病毒和 HIV p17 分析物存在,而 HIV 聚蛋白酶不存在。5 的结果表明登革热病毒和 HIV 聚蛋白酶存在,而 HIV p17 不存在。最后,6 的结果表明 HIV p17 和 HIV 聚蛋白酶存在,而登革热病毒不存在。如在本公开中其他地方描述的,这种编码方案可被扩大以编码额外的分析物。

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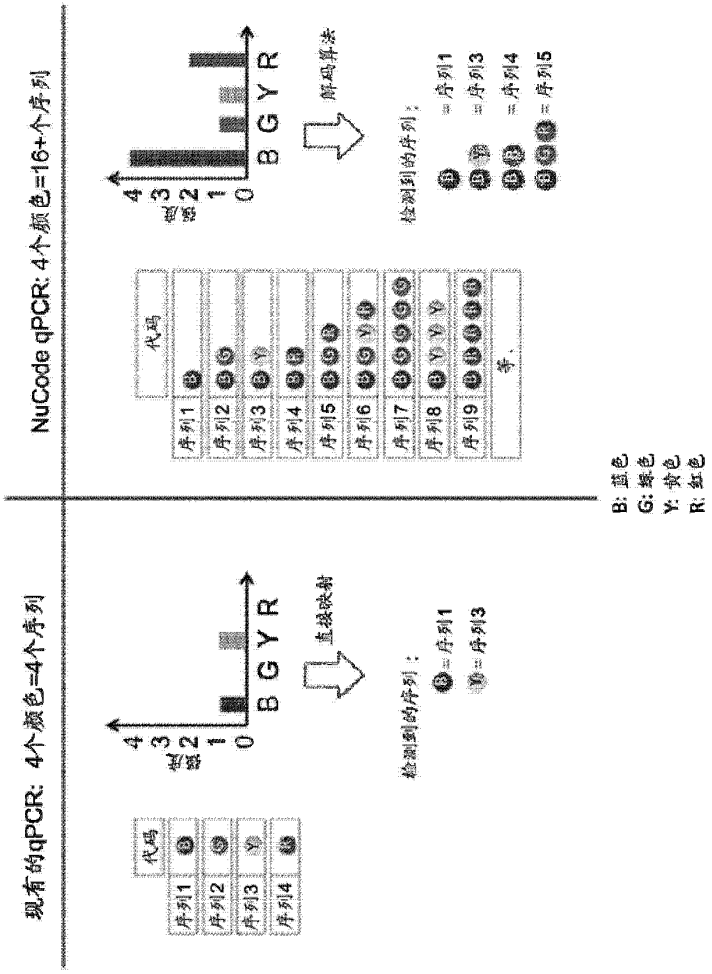


图 1

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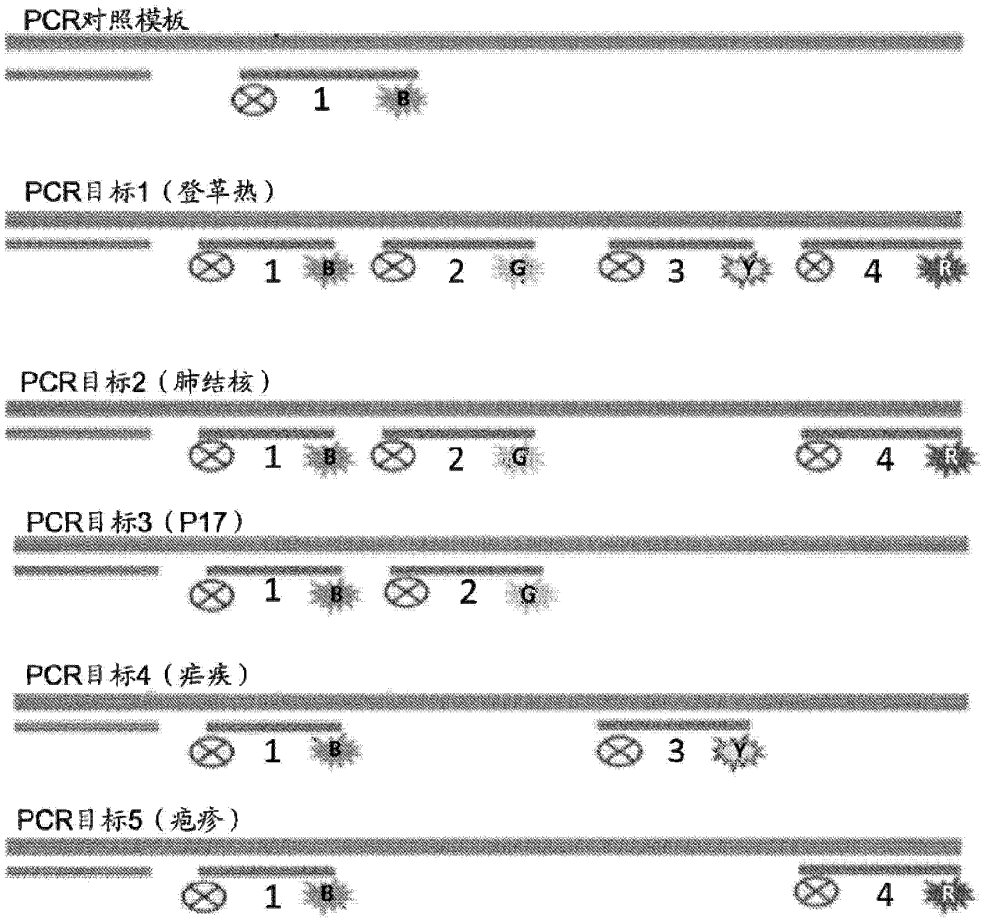


图 2

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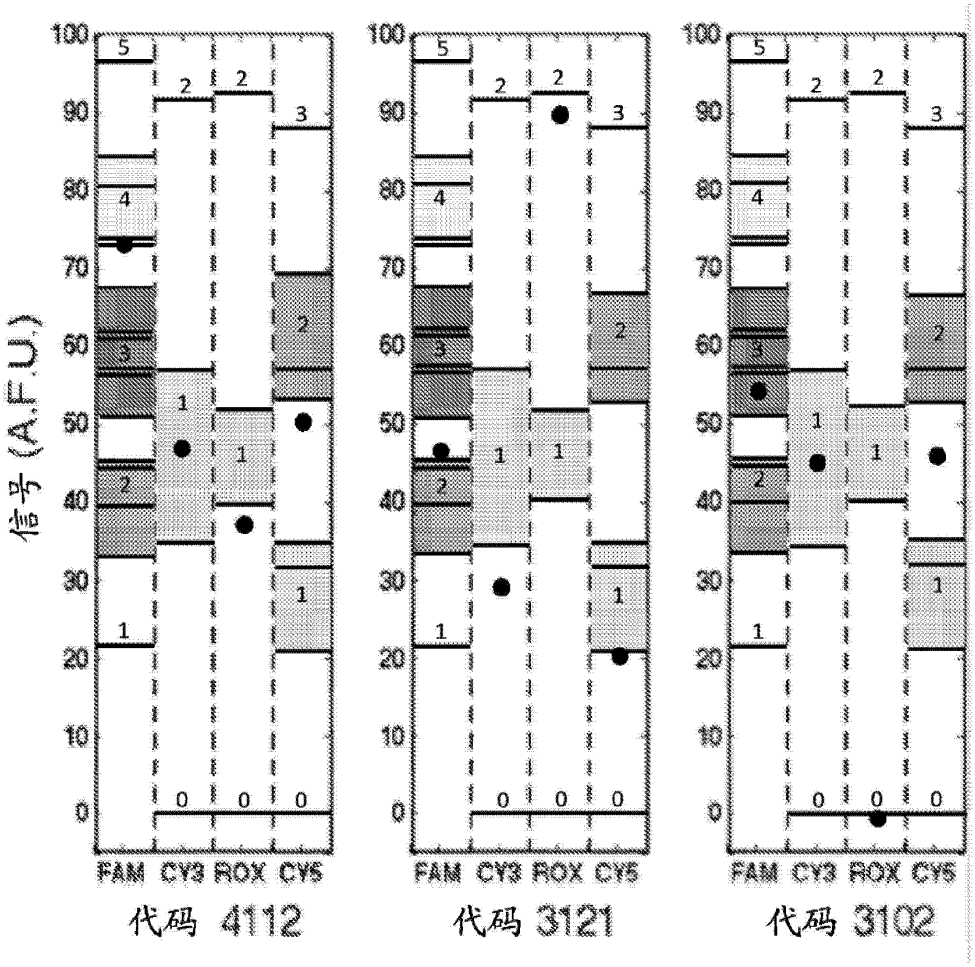


图 3

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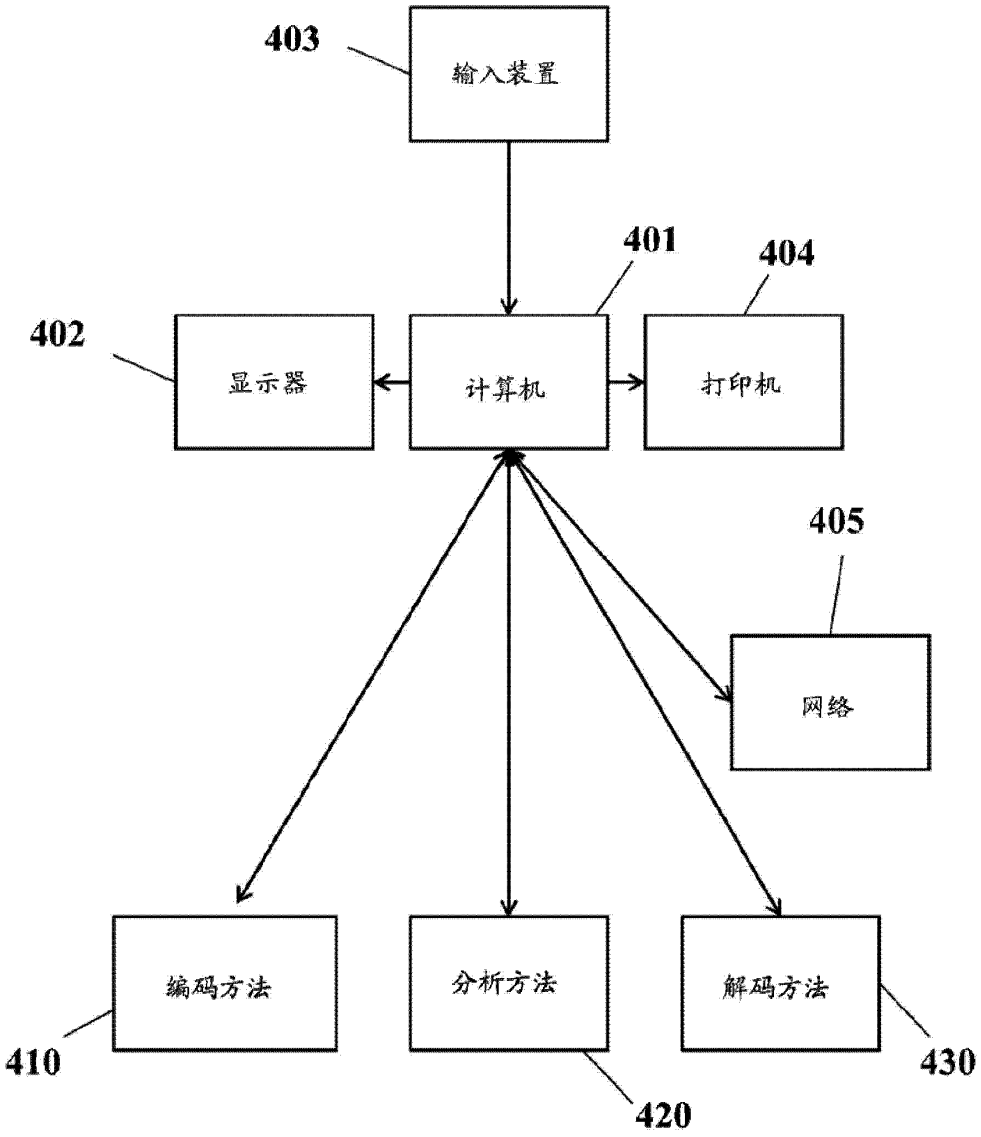


图 4

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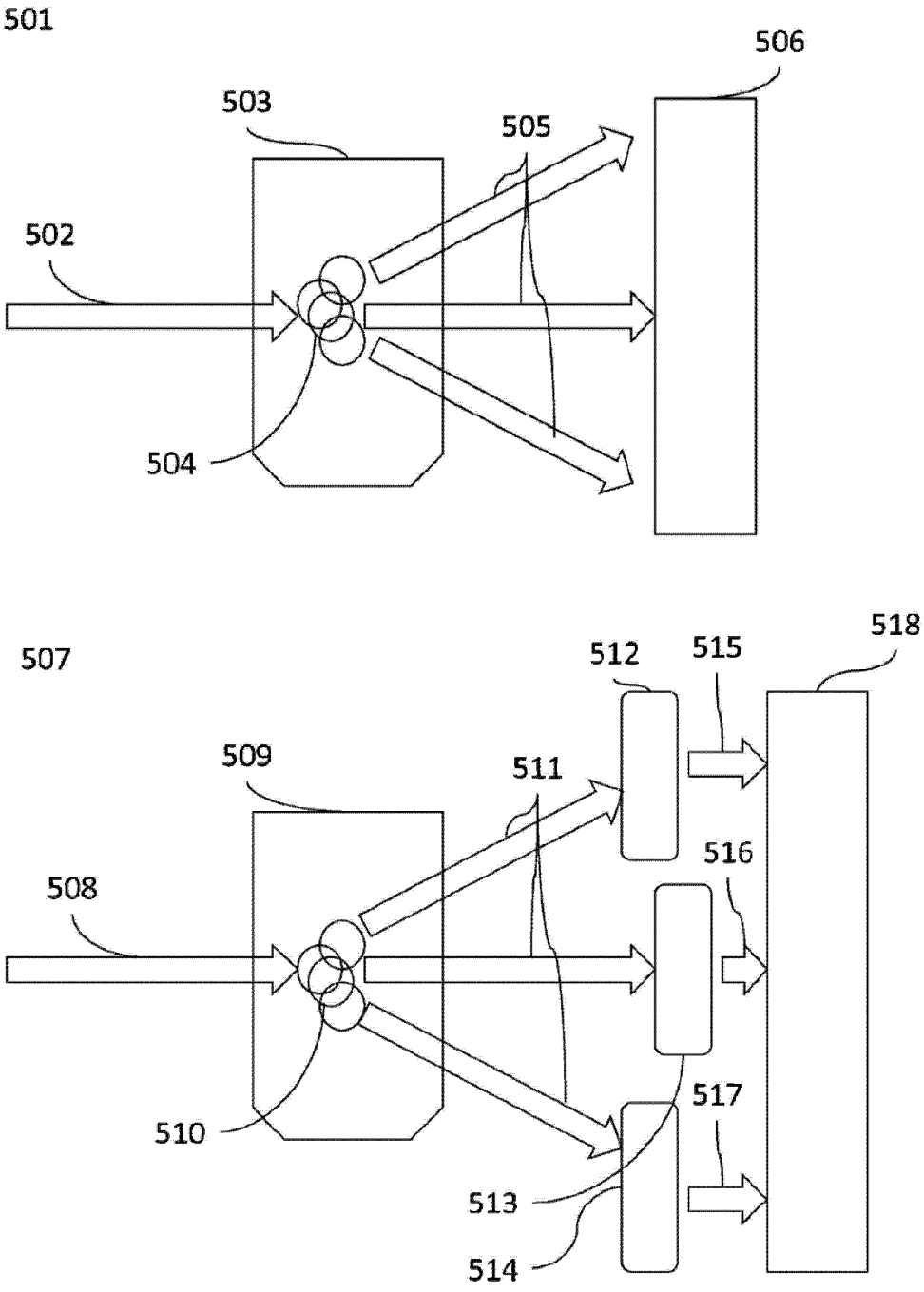


图 5

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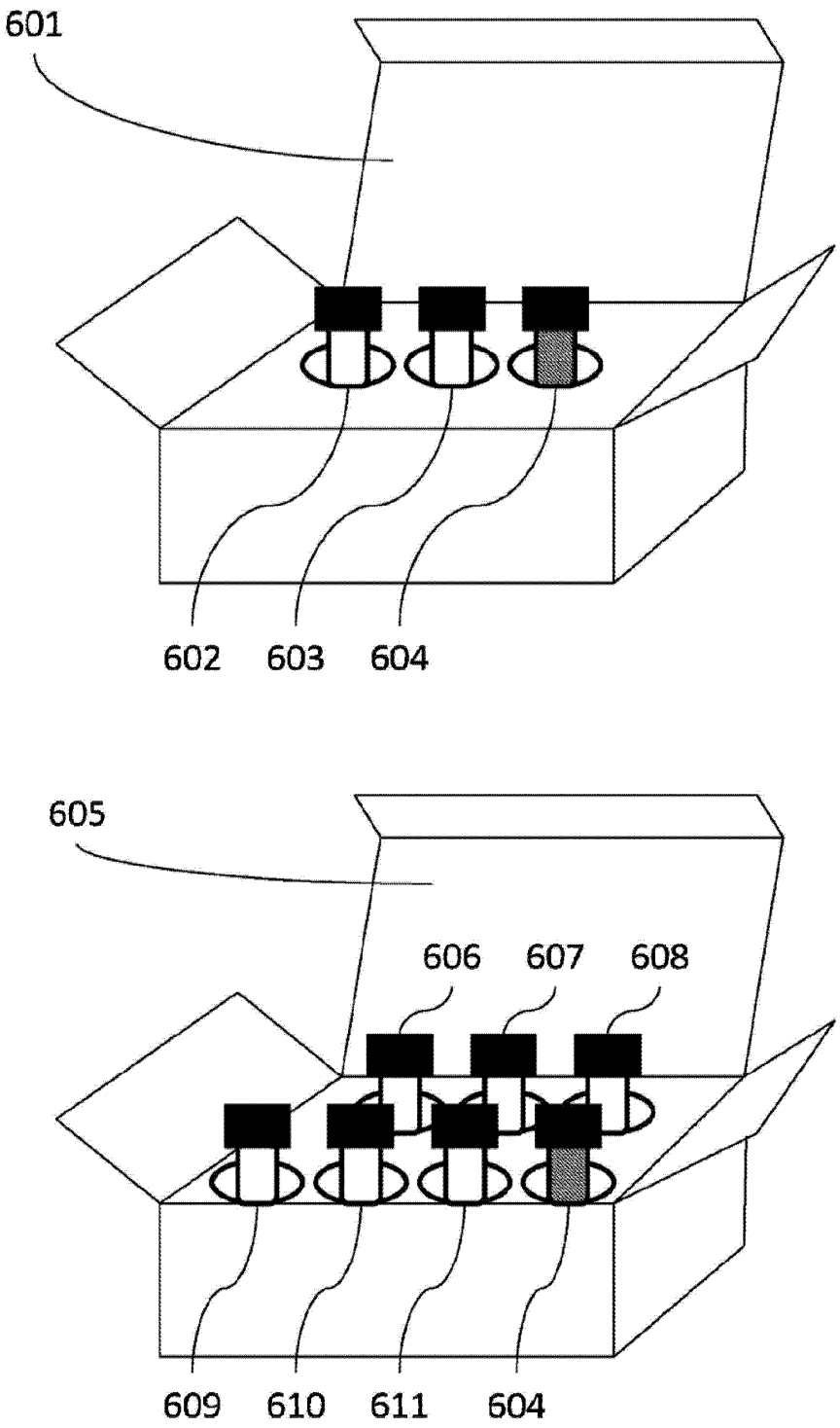


图 6

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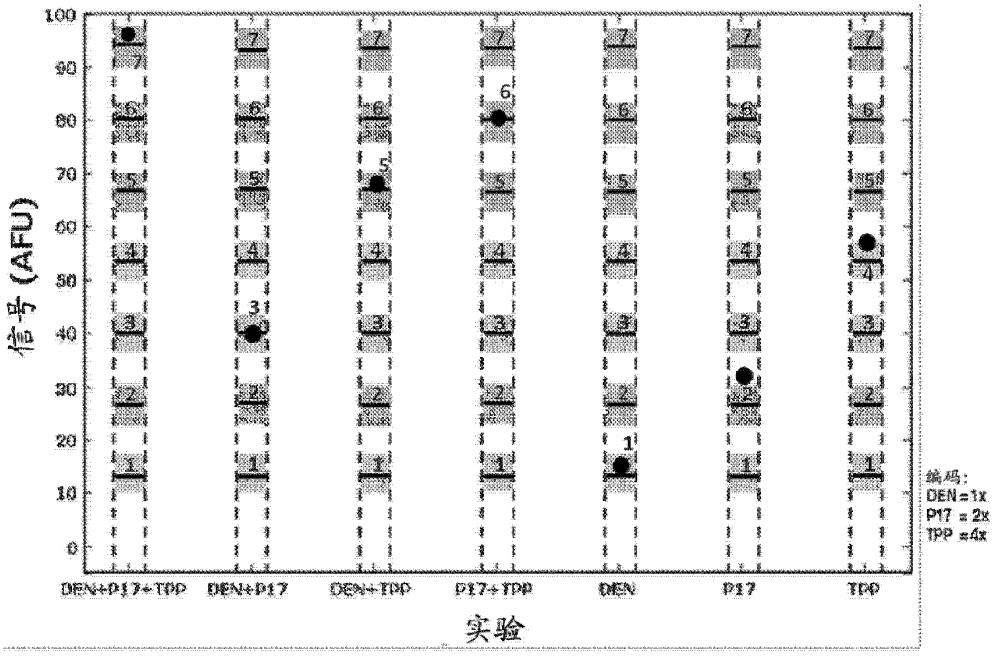
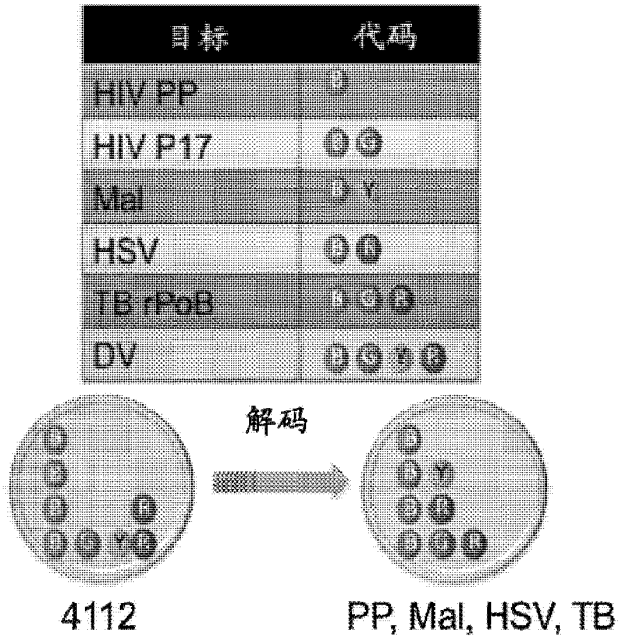


图 7

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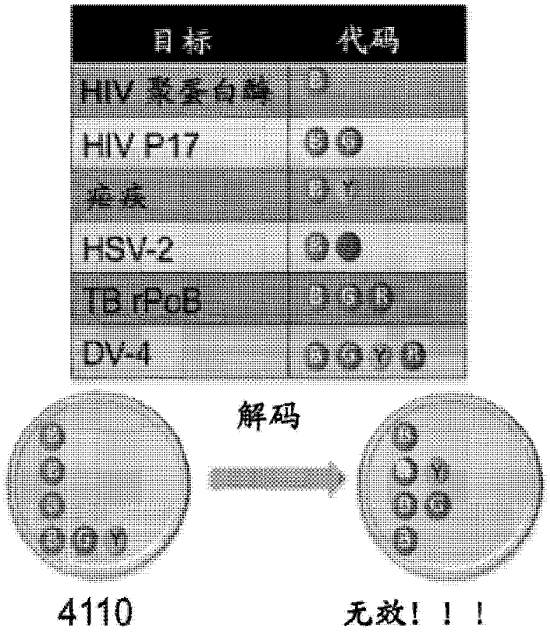


图 8

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DESCRIPTION CN107326085A

10 Encoding and decoding signals in multiplexed biochemical assays

[0001]

14 This application is a divisional application of an application filed on February 1, 2013, with application number 201380018760.9, and an invention titled "Encoding and Decoding of Signals in Multi-channel Biochemical Assays".

[0002]

20 cross reference

[0003]

24 This application claims the benefit of U.S. Provisional Application 61/594,480, filed on February 3, 2012, and U.S. Provisional Application 61/703,093, filed on September 19, 2012, each of which is incorporated herein by reference in its entirety.

[0004]

30 sequence list

[0005]

34 This instant application contains a sequence listing submitted in ASCII format via the EFS-Web page, and is incorporated herein by reference in its entirety.

36 The ASCII copy, created on February 14, 2013, is named 38075-716.601_SL.txt and is 9,240 bytes in size.

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[0006]

40 Technical field

[0007]

44 The present invention relates to the encoding and decoding of signals in multiplexed biochemical assays.

[0008]

48 Background technique

[0009]

52 Multiplex reactions offer significant advantages over traditional single-pass reactions, including performing multiple reactions in parallel on the same sample, performing more reactions using the same chamber, and extracting from the sample in a fast and efficient manner. The ability to enrich information.

55 However, to realize these advantages, multiplexed assays often require sophisticated reporting mechanisms, i.e., spectrally resolvable fluorescence or chemiluminescence (e.g., PCR, ELISA), spatially resolvable signals (e.g., microarrays, gel electrophoresis)), a temporally resolved signal (e.g., capillary electrophoresis), or a combination thereof (e.g., Sanger sequencing).

[0010]

62 Contents of the invention

[0011]

66 There is a need for multiplex reactions that can be performed in a single solution.

[0012]

70 The present disclosure provides methods, compositions, systems and kits for multiplex detection of analytes.

71 In some cases, the present disclosure provides assays that are capable of unambiguously detecting the presence or absence of in a single sample volume without performing immobilization, separation, mass spectrometry, or melting curve analysis. The presence or absence of each of at least 7 analytes in any combination.

74 In some examples, each analyte is encoded as the value of one (or at least one) component of the signal.

[0013]

78 In some examples, the present disclosure provides assays capable of unambiguously detecting the presence or absence of M analytes in any combination of presence or absence, where $M = \log2(F+1)$, and F is when all The

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maximum cumulative value of a component (eg, intensity) of a signal in the presence of an analyte.

[0014]

84 In some cases, each analyte is encoded as at least one first value in a first component of the signal (e.g., at least one intensity or intensity range) and at least one second value in a second component of the signal (e.g., at least one wavelength or range of wavelengths).

87 In some examples, each analyte is encoded as a first value in the first component of the signal for each of a plurality of second values in the second component of the signal (e.g., at a plurality of wavelengths or signal strength per wavelength range or signal strength range per wavelength range).

[0015]

93 In some examples, assays provided herein are capable of unambiguously detecting the presence or absence of M analytes in any combination of presence or absence, where $M = C*\log2(F+1)$, where C is is the number of second values encoding an analyte, and F is the maximum cumulative value of the first component of the signal for any second value in the presence of all analytes.

[0016]

100 In some cases, the present disclosure provides assays 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 code The number of codes per layer in the solution, and T is the number of layers.

103 In some cases, the number of layers $T=\log4(F+1)$, and F is the maximum cumulative value of the first component of the signal for any second value in the presence of all analytes.

[0017]

108 In some examples, the first value is intensity or intensity range.

109 In some cases, the encoding scheme includes at least three intensities or intensity ranges.

[0018]

113 In some cases, the second value is a wavelength or range of wavelengths.

114 In some examples, the encoding scheme includes at least five wavelengths or wavelength ranges.

[0019]

118 In some embodiments, the signal is an electromagnetic signal.

119 In some cases, the electromagnetic signal is a fluorescent emission signal.

120 In some embodiments, the intensity of the fluorescence emission signal at at least four wavelengths or ranges of wavelengths is measured.

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[0020]

125 In some cases, assays provided herein are performed using reagents that are lyophilized prior to use.

[0021]

129 In some cases, the detection step is performed using reagents including hybridization probes.

130 In some examples, the number of hybridization probes is greater than the number of analytes.

131 In some cases, a hybridization probe set includes one or more hybridization probes that are specific for different analytes and include the same fluorophore or combination of fluorophores.

133 In some examples, the sample is contacted with at least 18 of the hybridization probes.

134 In some examples, the detecting step is performed using a reagent that includes at least one pair of primers.

135 In some cases, at least one pair of primers is capable of amplifying a region complementary to at least three of said hybridization probes.

[0022]

140 In some examples, the measured signal is generated during a polymerase chain reaction.

141 In some cases, the polymerase chain reaction is selected from the group consisting of: end-point polymerase chain reaction, real-time polymerase chain reaction, digital polymerase chain reaction, and combinations thereof.

[0023]

147 In some examples, at least one cumulative measurement is performed on the solution.

[0024]

151 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: at least one additional value from the signal values of components, values of at least one component from different signals, and combinations thereof.

155 For example, at least one additional value may be selected from the group consisting of: fluorescence emission intensity, fluorescence emission wavelength, resonance energy transfer (FRET) emission intensity, FRET emission wavelength, electrochemical signal, chemiluminescence wavelength, chemiluminescence intensity, Fluorescent bleaching rate, chemiluminescent bleaching rate, and combinations thereof.

[0025]

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164 In some cases, chromatograms were constructed.

165 A chromatogram can be constructed by plotting all possible combinations of first and second values for positive control samples for each analyte.

[0026]

170 In some examples, at least one analyte includes a polynucleotide.

171 In some cases, the polynucleotide is from a source selected from the group consisting of animals, plants, bacteria, fungi, parasites, and viruses.

173 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, mycobacteria, dengue virus, hepatitis virus, and influenza virus.

176 In some cases, the polynucleotide is selected from the group consisting of human immunodeficiency virus polyprotease, human immunodeficiency virus p17, human papillomavirus E6, and human papillomavirus E7.

[0027]

182 In some cases, the sample is selected from the group consisting of clinical samples, food samples, environmental samples, pharmaceutical samples, and samples from consumer products.

[0028]

187 In some instances, information regarding the presence or absence of an analyte is transmitted over a computer network.

[0029]

192 In some cases, the physician is provided with information regarding the presence or absence of the analyte.

193 In some instances, clinical decisions are made based on such information.

[0030]

197 In some instances, at least one step of the methods provided herein is performed using instructions on a computer-readable medium.

199 In some cases, the instructions are located on a remote server.

200 In some instances, the instructions are located on the thermal cycler.

201 In some cases, the instructions are located on a computer that communicates with the thermal cycler.

[0031]

205 In some cases, at least one analyte is a positive control analyte.

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[0032]

209 In some instances, the encoding scheme is non-degenerate.

210 In some cases, encoding schemes are designed to be non-degenerate.

211 In some examples, the degeneracy is eliminated by enumerating every reasonable result that can be obtained from the coding scheme, identifying each reasonable result that is degenerate, and eliminating at least one potential analyte code from the coding scheme. The coding scheme is made non-degenerate.

[0033]

217 In some cases, the assays provided herein are endpoint assays.

218 In some examples, the present disclosure provides assays that end at a threshold number of cycles set by the detection limit of the instrument.

[0034]

223 In some examples, the assays provided herein are liquid phase assays.

[0035]

227 In some cases, the assays provided herein are quantitative.

[0036]

231 In some cases, the present disclosure provides a method of detecting the presence or absence of each of a plurality of analytes, the method comprising: (a) encoding each of said analytes as a first value of a signal, a coding scheme is thereby generated, wherein each said analyte is represented by said first value in said coding scheme, wherein said coding is performed in a manner that eliminates degeneracy; (b) providing a provision that includes or may include a sample of at least one of said analytes; (c) contacting said sample with an analyte-specific reagent that produces said first value in the presence of each of said analytes as represented in said coding scheme; (d) cumulatively measuring the first value of the signal in the sample, thereby providing a cumulative measurement; and (e) determining whether each of the analytes is present or present based on the cumulative measurement and the encoding scheme. does not exist.

[0037]

243 In some examples, the methods described in the previous paragraph are capable of unambiguously detecting the presence or absence of M analytes in any combination of presence or absence, where $M=\log2(F+1)$, and F is when all assays The maximum accumulated value of the first value when the object exists.

246 In some cases, the first value is an intensity or intensity range.

247 In some examples, the first value has a minimum value in the encoding scheme selected from the group

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consisting of: at least 1, at least 2, at least 4, at least 8, at least 16, at least 32, and at least 64.

249 In some cases, the first value is increased in the encoding scheme by an amount equal to the cumulative maximum of the previous first value plus one.

251 In other cases, the first value is increased in the encoding scheme by an amount greater than the cumulative maximum of the previous first value plus one.

[0038]

256 In some cases, the present disclosure provides methods of detecting the presence or absence of each of a plurality of analytes, the method comprising: (a) encoding each with at least one first value and at least one second value said analyte, 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, resulting in an encoding scheme wherein each said analyte represented by said at least one first value and said at least one second value in said encoding scheme, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (b) providing includes or may include at least one a sample of said analyte; (c) combining said sample with an analyte represented in said coding scheme that produces said at least one first value and at least one said second value in the presence of each said analyte specific reagent contact; (d) cumulatively measuring the first value and the second value in the sample, thereby providing a cumulative measurement; and (e) based on the cumulative measurement and the encoding scheme, Determining the presence or absence of each of said analytes, wherein when degeneracy is eliminated, the method is capable of unambiguously detecting in a single volume, when each of said reagents produces only one second value. The presence or absence of each of at least 6 analytes in any combination.

[0039]

273 In some cases, each analyte in the method described in the previous paragraph is encoded as a first in a first component of the signal for each of a plurality of second values in the second component of the signal. value.

275 In some cases, when degeneracy is eliminated, the method is able to unambiguously detect the presence or absence of each of at least 7 analytes in a single volume using 4 second values.

277 In some examples, when degeneracy is eliminated, the encoding scheme includes T non-degenerate layers, where $T=\log4(F+1)$, and F is the signal with respect to any second value when all analytes are present The maximum cumulative value of the first component.

280 In some cases, when degeneracy is eliminated, a method can unambiguously detect 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 layer.

283 In some examples, when degeneracy is eliminated, the method is able to unambiguously detect the presence or absence of M analytes in any combination of presence or absence, where $M = C*\log2(F+1)$, C is the number of second values in the encoding scheme, and F is the maximum cumulative value of the first component of the signal for any second value when all analytes are present.

[0040]

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290 The present disclosure also provides non-degenerate encoding schemes that are capable of unambiguously encoding, as present or absent, in a single sample volume without performing immobilization, separation, mass spectrometry, or melting curve analysis. The presence or absence of each of at least 7 analytes in any combination.

[0041]

297 In some examples, the non-degenerate coding scheme of the present disclosure is generated by a method including: (a) generating a code for each potential analyte, wherein each potential analyte passes at least one component of the signal encodes at least one value; (b) enumerates every reasonable cumulative result for all possible combinations of each analyte present or absent; (c) identifies every reasonable degenerate result; and (d) Eliminate at least one code to eliminate degeneracy.

302 In some cases, a coding scheme may be expanded by adding additional codes that are at least one unit greater than the sum of all previous codes in at least one component of the signal.

304 In some cases, coding schemes are generated by mathematical iterations where the structure guarantees non-degeneracy.

[0042]

309 In some cases, the present disclosure provides a system for detecting the presence or absence of each of a plurality of analytes, the system comprising: (a) encoding a first value for each of the analytes as a signal, thereby generating a coding scheme, wherein each said analyte is represented by said first value in said coding scheme, wherein said coding is performed in a manner that eliminates degeneracy; (b) providing a method including or a sample that may include at least one of said analytes; (c) combining said sample with an analyte-specific reagent that produces said first value when each said analyte is present, as represented in said coding scheme contact; (d) cumulatively measuring the first value of the signal in the sample, thereby providing a cumulative measurement; and (e) based on the cumulative measurement and the encoding scheme, determining each of the The analyte is present or absent.

[0043]

321 In some cases, the present disclosure provides a system for detecting the presence or absence of each of a plurality of analytes, the system comprising: (a) encoding with at least one first value and at least one second value for each of said analytes, 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, resulting in an encoding scheme, wherein each of said the analyte is represented by said at least one first value and said at least one second value in said coding scheme, wherein said coding is performed in a manner that reduces or eliminates degeneracy; (b) providing a method that includes or may include at least a sample of said analytes; (c) comparing said sample to said at least one first value and said at least one second value represented in said coding scheme when each said analyte is present. contacting an analyte-specific reagent of the value; (d) cumulatively measuring the first value and the second value in the sample, thereby providing a cumulative measurement; and (e) based on the cumulative measurement and the The encoding scheme determines the presence or absence of each of the

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analytes, wherein the method is capable of unambiguously detecting the presence or absence of each of the analytes in a single The presence or absence of each of at least 6 analytes in the volume in any combination of presence or absence.

[0044]

338 In some cases, the present disclosure provides methods for detecting the presence or absence of each of a plurality of analytes for a third group, the method comprising: (a) obtaining each of said analytes from the group identification; (b) encoding each of said analytes as a first value of a signal, thereby producing a coding scheme, wherein each of said analytes is represented by said first value in said coding scheme, wherein said coding is performed in a manner that eliminates degeneracy; (c) providing to said party the analyte specificity that yields said first value when each said analyte is present, as represented in said coding scheme a reagent that: (i) contacts a sample that includes or may include at least one of said analytes with said reagent; and (ii) cumulatively measures said first value in said sample, thereby providing a cumulative measurement results; and (d) obtaining said cumulative measurements from said group; (e) determining whether each of said analytes is present or absent based on said cumulative measurements and said coding scheme; and (f) placing Information regarding the presence or absence of each of the analytes is provided to the group.

[0045]

352 In some cases, the present disclosure provides methods for detecting the presence or absence of each of a plurality of analytes for a third group, the method comprising: (a) obtaining each of said analytes from the group identification; (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 the signal, and said second value is a value from the second component of said signal resulting in a coding scheme, wherein each said analyte is represented in said coding scheme by said at least one first value and said at least one second value, wherein said coding is performed in a manner that reduces or eliminates degeneracy; and (c) providing said party with the results of generating said at least one first value in the presence of each of said analytes and said at least one second value of an analyte-specific reagent, said body: (i) contacting a sample that includes or may include at least one said analyte with said reagent; and (ii) cumulatively measuring said sample the first value and the second value, thereby providing a cumulative measurement; and (d) obtaining the cumulative measurement from the group; (e) based on the cumulative measurement and the encoding scheme, determining each the presence or absence of each of the analytes; and (f) providing information to the party regarding the presence or absence of each of the analytes.

[0046]

369 In some cases, the present disclosure provides compositions for detecting the presence or absence of each of a plurality of analytes, the compositions comprising analyte-specific reagents, each reagent producing a value including a first A signal wherein the reagent is capable of unambiguously detecting, in a single sample volume, any combination of presence or absence of at least 7 The presence or absence of each analyte.

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[0047]

376 In some cases, the present disclosure provides compositions for detecting the presence or absence of each of a plurality of analytes, the compositions comprising an analyte-specific reagent, each reagent producing a composition including at least one first a signal of one value and at least one second value, the at least one first value being a value from a first component of the signal and the at least one second value being a value from a second component of the signal, wherein , said reagents are capable of detecting the presence of each of at least 6 analytes in any combination of presence or absence in a single volume, when each said reagent produces only one second value, or does not exist.

[0048]

386 In some cases, the present disclosure provides kits for detecting the presence or absence of each of a plurality of analytes, the kits comprising analyte-specific reagents, packaging, and instructions, each reagent Generating a signal that includes a first value, wherein the kit is capable of unambiguously detecting the presence or absence of any analyte in a single sample volume without performing immobilization, separation, mass spectrometry, or melting curve analysis. The presence or absence of each of at least 7 analytes in combination.

[0049]

395 In some examples, the present disclosure provides kits for detecting the presence or absence of each of a plurality of analytes, the kits comprising analyte-specific reagents, packaging, and instructions, each reagent Generating a signal comprising at least one first value from a first component of the signal and at least one second value from a second component of the signal value of a component, wherein the kit is capable of detecting at least 6 analytes in any combination of presence or absence in a single volume when each of said reagents produces only one second value The presence or absence of each analyte.

[0050]

404 In some cases, the present disclosure provides a kit for detecting the presence or absence of each of a plurality of analytes, the kit comprising a cartridge body 601, a card disposed in the cartridge body for placing a vial. A vial 602 including a probe for detection of an analyte, a vial 603 including a primer for amplification, and a vial 604 including a reagent for amplification. The kit can be used at different times. Unambiguously detecting the presence of each of at least 7 analytes in any combination of presence or absence in a single sample volume while performing immobilization, separation, mass spectrometry, or melting curve analysis or does not exist.

[0051]

414 In some examples, the present disclosure provides a kit for detecting the presence or absence of each analyte in a plurality of analytes, including: a box body 601, and a snap-in slot arranged in the box body for placing a

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bottle. , a vial 602 including a probe for detection of the analyte, a vial 603 including a primer for amplification, and a vial 604 including reagents for amplification, each probe generating a probe including at least a first value and a signal of at least one second value, the at least one first value being a value from a first component of the signal and the at least one second value being a value from a second component of the signal, wherein when each The kit is capable of detecting the presence or absence of each of at least 6 analytes in any combination of presence or absence in a single volume when each of said probes produces only one second value. exist.

[0052]

426 Incorporated by reference

[0053]

430 All publications and patent applications mentioned in this specification are hereby 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.

[0054]

436 Description of the drawings

[0055]

440 The novel features of the invention are set forth with particularity in the appended claims.

441 A better understanding of the features and advantages of the invention will be obtained by reference to the following detailed description of illustrative embodiments in which the principles of the invention are utilized, and in which are the accompanying drawings:

[0056]

447 Figure 1 shows a comparison between a conventional encoding method that detects four analytes in four colors, and the encoding method of the present invention that is capable of detecting 16 or more sequences in four colors.

450 Colors are indicated by B, G, Y and R, which indicate blue, green, yellow and red respectively.

[0057]

454 Figure 2 shows a schematic representing the detection of 6 analytes (including controls) in four colors.

455 Analytes are detected using hybridization probes attached to fluorophores (B, G, Y, R; blue, green, yellow, and red, respectively) and quenchers (ovals marked with an "X").

457 The control sequence and five other sequences (Dengue = Dengue virus; Tuberculosis = Mycobacterium

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tuberculosis; P17 = HIV p17; Malaria = Plasmodium falciparum; and Herpes = Herpes simplex virus 2) were labeled using the Probe detection with blue fluorophore.

460 Each non-control analyte was detected using 1-3 additional probes.

461 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 an additional probe with a red fluorophore; and so on.

[0058]

467 Figure 3 shows a chromatogram of experimental results obtained as described in Example 2.

[0059]

471 Figure 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.

[0060]

476 Figure 5 shows a schematic diagram of two different methods of detecting the wavelength and intensity of a light signal, such as a fluorescence emission signal.

[0061]

481 Figure 6 shows a schematic diagram of an exemplary kit.

[0062]

485 Figure 7 shows a chromatogram of experimental results obtained as described in Example 3.

[0063]

489 Figure 8 shows a schematic diagram of reasonable results (top) and unreasonable results (bottom).

[0064]

493 Detailed ways

[0065]

497 Due to several desirable properties, including compatibility with biochemical assays, relatively small size of the fluorescent label, simple means of conjugating molecules of interest, affordability, low toxicity, stability, robustness, inexpensiveness to utilize Due to the detectability of optical systems and the ability to combine

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with spatial arrays, fluorescence detection has become the preferred technology for multiplex determination.

501 However, standard fluorescence has a broad emission spectrum.

502 Therefore, to avoid spectral overlap (i.e., to maintain spectral resolution) only a relatively small number of colors (eg, 4 to 6) are typically used simultaneously in multiplexed fluorometry.

[0066]

507 Traditional coding methods for multiplexed fluorometric assays have been to code each analyte with a single color; that is, $M=N$, where M is the number of detectable analytes and N is the number of spectrally resolvable fluorescent probes.

510 Whenever higher multiplexing factors are required (i.e., $M>N$), fluorescence is often combined with other technical techniques, such as aliquoting, spatial arraying, or sequential processing.

512 These additional processing steps are labor intensive and often require relatively expensive and complex optical and mechanical systems (e.g., spectrometers, mechanized microscope stages, microfluidics, droplet generators, scanners, and similar systems).

515 Such systems are often impractical to configure in certain environments, particularly point-of-care and low-resource settings.

517 Therefore, there is a significant need for multi-pass encoding and decoding methods that can provide cheap multi-pass means while avoiding the use of expensive additional processing steps.

[0067]

522 The present disclosure provides methods, systems, compositions, and kits for detection of multiple analytes in a sample.

524 This article presents methods for detection of analytes based on encoding, analysis, and decoding.

525 In some examples, each analyte to be detected is encoded as a value of a signal (eg, intensity), where the value is assigned such that the result of the assay unambiguously indicates the presence or absence of the analyte being assayed.

528 In other examples, each analyte to be detected is encoded as a value in each of at least two components of the signal (eg, intensity and wavelength).

530 At least two components of the signal can be orthogonal.

531 Similarly, as described more fully elsewhere in this disclosure, multiple orthogonal signals may be used, such as a combination of fluorescent and electrochemical signals.

533 The analyte can be any suitable analyte, such as polynucleotides, proteins, small molecules, lipids, carbohydrates, or mixtures thereof.

535 The signal may be any suitable signal, such as an electromagnetic signal, an optical signal, a fluorescent emission signal, an electrochemical signal, a chemiluminescent signal, and combinations thereof.

537 The at least two components of the signal may be any suitable two components, such as amplitude and frequency, or intensity and wavelength.

[0068]

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542 After encoding the analytes, a sample is provided, wherein the sample includes or may include at least one of the encoded analytes.

544 The sample is contacted with one or more analyte-specific reagents specified for each analyte in the coding scheme that produce a specific signal in the presence of the analyte.

546 The reagent may be any suitable reagent capable of generating such a signal in the presence of its corresponding analyte, for example, an oligonucleotide probe (eg, a TAQMAN probe) linked to a fluorophore and a quencher.

549 If the reagents are oligonucleotide probes linked to a fluorophore and a quencher, nucleic acid amplification that produces a signal can be performed.

[0069]

554 After adding reagents, the signal is quantified.

555 In some cases, this 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 value encoding the presence of each analyte, and the cumulative value of the signal. kind of quantitative.

[0070]

561 In some cases, at least two components of the signal (eg, intensity and wavelength) are measured cumulatively over the sample.

563 This measurement may be performed, for example, by measuring the intensity at a specific wavelength, or the intensity within a specific range of wavelengths.

565 The presence or absence of the analyte may then be determined based on the value of each of the at least two components of the signal and the values used to encode the presence of the analyte (ie, those values in the encoding scheme).

[0071]

571 Coding may be performed in a manner that reduces or eliminates the number of possible degenerate (eg, ambiguous) results obtained by the method.

573 As described elsewhere in this specification, the complete coding capabilities 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.

576 Likewise, encoding schemes can be designed to be non-degenerate so that reduction or elimination of degeneracy is not necessary.

578 A decoding matrix can be constructed to correspond to the constituent signals of the accumulated signal measurements that interpret the accumulated signal measurements (eg, intensity or intensity at a specific wavelength) as the presence or absence of certain analytes.

[0072]

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584 I.

585 definition

[0073]

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

[0074]

594 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.

596 In addition, for the terms "including", "includes", "having", "has", "with", "such as", or variations thereof When used in the specification and/or claims, these terms are not limiting and are intended to be inclusive in a manner similar to the term "comprising".

[0075]

602 The term "about" as used herein generally refers to a range of 15% more or less than 15% from the numerical value stated in the context of the particular usage.

604 For example, "about 10" would include the range from 8.5 to 11.5.

[0076]

608 The terms "dimension" and "component" as used herein, when referring to a signal, generally refer to aspects of the signal that can be quantified.

610 For example, if a signal is generated by a fluorescent molecule, it can be quantified in terms of its wavelength (eg, first dimension or component) and its intensity (eg, second dimension or second component).

[0077]

615 The term "decoding," as used herein, generally refers to a method of determining which analyte is present based on a cumulative signal and an encoding scheme or decoding capable of converting the cumulative signal into information regarding the presence or absence of one or more analytes. matrix.

[0078]

621 The term "encoding", as used herein, generally refers to the processing of an analyte using a method that includes a value of a signal (e.g., intensity), or a value of each of at least two components of a signal or signals (e.g., wavelength and intensity) codes.

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[0079]

627 The terms "oligonucleotide probe linked to a fluorophore and quencher" and "TAQMAN probe" generally refer to hydrolysis probes used in polynucleotide amplification assays to detect the presence of analytes.

629 These probes include oligonucleotide probes linked to fluorophores and quenchers.

630 As long as the quencher and the fluorophore are in close proximity, the quencher will quench the fluorescence emitted by the fluorophore when excited by the light source.

632 The sequence of the oligonucleotide probe is designed to be complementary to the polynucleotide sequence present in the analyte and, therefore, capable of hybridizing to the polynucleotide sequence present in the analyte.

635 Hybridization of oligonucleotide probes is performed in a nucleic acid amplification reaction (eg, polymerase chain reaction) including primers.

637 After extension of the primer by DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and quencher into the medium.

639 The proximity between the fluorophore and the quencher is destroyed, and the signal from the fluorophore is no longer quenched.

641 Therefore, 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 the analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be generated.

[0080]

647 The term "orthogonal" as used herein generally refers to at least two components of a signal (e.g., wavelength and intensity) that can vary independently or approximately independently, or at least two distinct signals (e.g., a fluorescence emission signal and a electrochemical signal).

650 For example, when working with fluorescent molecules, wavelength and intensity are considered to be orthogonal or nearly orthogonal. The wavelength of fluorescence emission will depend on, among other factors, the composition of the fluorescent molecules, and the intensity of the fluorescence will depend on the amount of molecules 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. It is also possible to use components of a signal that vary independently, or multiple signals, as long as the components or signals are characterized well enough to be able to be encoded, measured and decoded. For example, if a change in one component or signal affects a change in another component or signal, both components or signals can still be used as long as the relationship between the changes is understood.

[0081]

663 The terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to a biological molecule that includes a plurality of nucleotides.

665 Exemplary polynucleotides include deoxyribonucleic acid, ribonucleic acid, and synthetic analogs thereof, including peptide nucleic acids.

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[0082]

670 The term "probe" as used herein generally refers to an agent capable of generating a signal in the presence of a specific analyte.

672 A probe usually has at least two parts: a part that can specifically recognize the analyte, or a part thereof; and a part that can generate a signal in the presence of the analyte, or a part thereof. As described above and elsewhere in this disclosure, the probe may be an oligonucleotide probe linked to a fluorophore and a quencher. A probe can also be any reagent that generates a signal in the presence of an analyte, such as an antibody that detects the analyte, with a fluorescent label that emits or is quenched by binding of the antibody to the analyte. Any suitable probe can be used in the methods set forth in this disclosure, so long as the probe produces a quantifiable signal in the presence of the analyte. For example, the analyte-specific portion of the probe can be coupled to an enzyme that converts an uncharged substrate into a charged product in the presence of the analyte, thereby increasing the conductivity in the medium over time. . In this case, different analytes can be encoded in different ratios by coupling the analyte-specific portion of the probe (eg, hybridization probe or antibody) to the enzyme. The rate at which increased conductivity in the medium is produced will accumulate for all analytes present in the medium. According to the methods provided herein, encoding analytes is capable of converting conductivity measurements into unambiguous (i.e., non-degenerate) results that provide information regarding the presence or absence of a specific analyte. Similarly, the probe may include an enzyme that produces a chemiluminescent product from a substrate. The amount of chemiluminescence can then be used to encode the presence of a specific analyte.

[0083]

691 II.

692 Encoding and decoding methods

[0084]

696 A.

697 Traditional fluorescence encoding and decoding methods

[0085]

701 A common method for determining the presence of an analyte is to use four spectrally resolvable fluorescent molecules to indicate the presence or absence of four analytes.

703 The left-hand side of Figure 1 shows an example of this approach. The encoding method is shown on the left-hand side of Figure 1, where the four analytes (Sequence 1, Sequence 2, Sequence 3, and Sequence 4) are each encoded by a single color (blue, green, yellow, and red, respectively). Colors represent fluorophores attached to oligonucleotide probes that also include quenchers. In the system shown on the left hand side of Figure 1, there are four different oligonucleotide probes, each including a single fluorophore (blue, green, yellow, or red) and a quencher. The presence or absence of the analyte is determined based on the presence or absence of signal in a particular color.

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[0086]

713 The graph on the left hand side of Figure 1 shows intensity versus color for a hypothetical sample containing two analytes: Sequence 1 and Sequence 3.

715 The presence of these analytes is determined based on measurements of the blue signal (corresponding to sequence 1) and the yellow signal (corresponding to sequence 3). The absence of sequence 2 and sequence 4 is indicated by the absence of green and red signals.

[0087]

721 Table 1 shows the interpretation of the binary format for this encoding scheme.

722 Each analyte is encoded as a value in each of two components of the fluorescence signal: (1) color (also called wavelength; or wavelength range) and (2) intensity (indicated by numbers in the table) . For example, A (eg, sequence 1) has a color of blue and an intensity of 1; B (eg, sequence 2) has a color of green and an intensity of 1; and so on. Quantify the intensity of the signal within each color range as described herein, for example, by measuring the intensity of the signal within a specific wavelength range as determined by a bandpass filter. A result of 1000 indicates that only analyte A is present; a result of 1100 indicates that both analytes A and B are present; and so on.

[0088]

732 Table 1.

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

[0089]

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

[0090]

742 B.

743 Coding methods using more than one color per analyte

[0091]

747 The traditional method described above suffers from the fact that it is limited by the number of spectrally resolvable fluorophores.

749 More specifically, the number of detectable analytes is equal to the number of spectrally resolvable fluorophores.

751 Therefore, the number of analytes can only be increased by increasing the number of spectrally resolvable

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fluorophores.

[0092]

756 The present disclosure provides methods to overcome this limitation.

757 More specifically, in some cases, the methods described herein can be used to detect more than one analyte per fluorophore by utilizing at least two components of the signal during encoding. For example, using the methods provided in this article, each fluorophore can detect 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. In some cases, the methods provided herein can 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, per fluorophore. 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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. In some cases, the methods provided herein can be used to detect 1.5-2, 2-4, 1.5-4, 4-6, 2-6, 6-10 analytes per fluorophore.

[0093]

769 In some cases, methods provided by the present disclosure may include the use of contrasting colors.

770 The control color can be attached to one or more probes that bind the positive control analyte, and each analyte to be detected in the sample. A single control probe can also be used if the same sequence occurs in the positive control analyte and in each analyte to be detected. If the same sequence does not occur in the positive control analyte and in each analyte to be detected, different probes can be used, but each probe can still be connected to the control color.

[0094]

778 For example, building on the traditional approach described above, a color (eg blue) can be used to encode the presence of a control analyte that is always present in the sample.

780 Control analytes may be added to the sample or may be inherently present in the sample. The remaining colors (eg, green, yellow, red) can be used to encode the presence of additional analytes. Table 2 shows an example of such an approach.

[0095]

786 Table 2.

787 Four analytes (including controls) are coded in four colors and up to two colors per analyte.

[0096]

791 Blue Green Yellow Red Control (p) 1 0 0 0 A 1 0 0 1 B 1 0 1 0 C 1 1 0 0

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[0097]

795 In Table 2, the presence of a control group (p) is indicated by a result of 1000.

796 The presence of control and analyte A is indicated by the results of 2001.

797 The presence of the control and all other three analytes is indicated by a result of 4111. The control color (blue) provides an indication of normal operation of the assay. The intensity of the blue color reports the number of analytes present in the test sample. Of course, any color can be used as a contrasting color. One skilled in the art will recognize that certain practical considerations may make it preferable to use one color over another as a contrasting color. For example, if a color is better detected in a particular optical system (or fluorophore system), then it may actually be preferable to use that color as a control color.

[0098]

806 The coding scheme shown in Table 2 codes each analyte using a control color and an additional color (up to 2 probes per analyte).

808 However, as shown below, when using up to 4 colors per analyte, the number of analytes that can be encoded increases. Table 3 shows an exemplary coding scheme, where each analyte is coded by up to 4 colors. In the protocol 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). Each of the other seven analytes is coded by the presence of one to three additional colors. Colors may be comprised of different probes (eg, oligonucleotide probes linked to a fluorophore and a quencher) or the same probe (which can have multiple fluorophores and multiple quenchers).

[0099]

818 table 3.

819 Eight analytes (including controls) are coded in four colors and up to four colors per analyte.

[0100]

823 Blue Green Yellow Red Control (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

[0101]

827 In Table 3, the presence of the control and all other seven analytes is indicated by a result of 8444.

828 Three analytes (A, B, and C) are coded by two colors.

829 Three analytes (D, E, and F) are coded by three colors, and one analyte (G) is coded by four colors. The presence of control (p) is indicated by a result of 1000. The presence of control and analyte A is indicated by the results of 2001. The presence of control and analyte G is indicated by the result 2111. These results express the cumulative intensity of the signal in each color. For example, result 2111 has a signal strength of 2X in the blue channel, while result 1000 only has a signal strength of 1X in the blue channel.

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[0102]

837 Using Table 3, each possible cumulative measurement result can be enumerated according to color (blue, green, yellow, red) and intensity (0-8).

839 Table 4 shows the "decoding" generated by enumerating each possible cumulative assay result based on the encoding method shown in Table 3 and providing the corresponding decoding result for each assay based on the analytes present in the sample. matrix". Codes for any of the coding schemes described herein can be generated by enumerating each possible cumulative assay result based on the coding scheme and providing a corresponding decoding result for each assay based on the presence (or absence) of the analyte in the sample. Similar decoding matrix. In some cases, as described below, one or more analytes may be removed from the coding scheme to reduce or eliminate degeneracy.

[0103]

849 Table 4.

850 The decoding matrix for the encoding method shown in Table 3.

[0107]

854 The decoding matrix is provided in Table 4 constructed using two assumptions.

855 First, the decoding matrix assumes that the positive control (P) always produces a positive result.

856 Second, the decoding matrix assumes that within each color, regardless of which probe the signal may come from, the intensity is added and scaled in the same way as changing the probe concentration.

858 This essentially means that the signal is additive and digital.

859 The conditions on which these assumptions are based can be satisfied by appropriate preparation of the assay. As demonstrated in the examples provided herein, if fluorescent signals are used, the intensity need only be approximately additive and numerical.

[0108]

865 Table 4 allows cumulative measurements of intensity in four ranges of fluorescence wavelengths (ie, signal intensity in each color range) to be converted to the corresponding analytes present in the sample.

867 For example, a result of 4321 indicates the presence of p, C, F, and G, and the absence of the other analytes. This result is called a "reasonable" result because it appears in the decoding matrix. In contrast, the result of 4000, although possibly measurable, does not appear in the decoding matrix. More specifically, a result of 4000 cannot be achieved by adding any combination of control codes and analyte codes from Table 3. This result is called an "unreasonable" result. Unreasonable results may indicate an assay failure. Therefore, the control (p) and decoding matrices provide a means of verifying that the assay is functioning properly.

[0109]

876 Table 4 enumerates any combination of fluorophores that produces four resolvable emission spectra (eg, colors).

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878 The term "rank" is used to describe the number of analytes detected including controls. In the example provided above, if the assay is functioning properly, the rank is equal to the value of the blue signal. For example, a rank of 8 indicates that the control and all other seven analytes (A-G) are present. Rank 2 indicates the presence of a control and only one analyte. The lowest rank is rank 1, which indicates that only controls are present. The number of possibilities at each rank can be enumerated. For example, continuing with reference to the encoding and decoding methods described in Table 3-4, there are 7 possibilities for rank 2. The number of possibilities at rank 3 can be calculated as a combination of 2 out of 7 or there are 7!/(5!*2!) = 21 possibilities. More generally, the number of possibilities is the combination of N and K, which is N!/((N-K)!*K!). Similarly, at ranks 4, 5, 6, 7 and 8, the number of possibilities are 35, 35, 21, 7 and 1 respectively. Reference to Table 4 shows that the table is consistent with theoretical predictions. Therefore, Table 4 is an exhaustive decoding matrix for the encoding methods provided in Table 3.

[0110]

892 C.

893 Reduce or eliminate degeneracy

[0111]

897 The terms "degeneracy" and "degeneracy," as used herein, generally describe situations in which a reasonable outcome is ambiguous because it may indicate more than one possibility depending on the presence or absence of the analyte.

900 For example, referring to Table 4, the result 5233 is degenerate because it can be decoded as pADFG or pBDEG. Similarly, the result 4222 is degenerate because it can be decoded into any of the following: pCDG, pAFG, pBEG, pDEF. In contrast, result 3110 can only indicate pBC and is therefore non-degenerate.

[0112]

906 The present disclosure provides methods to reduce or eliminate degeneracy, thereby increasing the confidence with which analytes are detected.

908 In one embodiment, the method of eliminating degeneracy includes: (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 the signal the value of analyte codes), wherein eliminating the at least one potential analyte reduces or eliminates degeneracy. For example, referring to the encoding scheme described in Table 3, and the decoding matrix described in Table 4 (enumerating every reasonable result), eliminating any two of analytes D, E, and F eliminates degeneracy. Eliminating any of analytes D, E, and F will not eliminate the degeneracy, but it will reduce it.

[0113]

918 Continuing with the coding scheme described in Table 3, eliminate any two of analytes D, E, and F from the coding scheme, resulting in a scheme that can analyze 6 analytes (including controls) using only 4 colors

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without degeneracy. .

921 In contrast, a multiplexed conventional method using 4 colors would only allow reporting of 3 analytes and 1 control. Therefore, the number of analytes that can be analyzed by using the methods provided herein is nearly doubled.

[0114]

927 Figure 2 shows an exemplary embodiment of the invention where 4 colors are used to detect five analytes and one control.

929 Analytes are derived from dengue virus ("Dengue"), Mycobacterium tuberculosis ("TB"), human immunodeficiency virus (HIV) p17 (P17), Plasmodium ("Malaria"), and herpes simplex ("Herpes") nucleic acid. The "color" in this example is a fluorophore attached to an oligonucleotide probe that also includes a quencher. Oligonucleotide probes will usually be different for different analytes while being the same color. For example, all probes designated as Probe 1 have a blue color but will typically have different oligonucleotide sequences. Of course, the probe designated as probe 1 can also have the same oligonucleotide sequence if complementary sequences are present in each analyte. The mechanism of detection with these probes is described elsewhere in this disclosure. Probe 1 (blue) hybridizes to all 6 analytes including the control. Probe 2 (green) hybridizes analytes from Dengue virus, Mycobacterium, and HIV p17. Probe 3 (yellow) hybridizes analytes from dengue virus and Plasmodium. Probe 4 hybridizes analytes from Dengue virus, Mycobacterium, and Herpes simplex. The coding scheme defined by these probes is shown in Table 5. Referring to Table 3, potential analytes D (1011) and F (1110) have been eliminated from the coding scheme. Therefore, the coding scheme shown in Table 5 is non-degenerate, and each reasonable result from the assay corresponds to the presence or absence of a unique combination of analytes in the sample.

[0115]

946 table 5.

947 Coding schemes for the detection of dengue virus, mycobacteria, HIV, Plasmodium, and herpes simplex are illustrated in Figure 2.

[0118]

952 The methods shown above for encoding and decoding, including methods for eliminating degeneracy, apply equally to encoding methods using additional colors.

954 For example, Table 3 could be expanded by including additional colors and additional intensities (described further below).

956 Then, as mentioned above, the enumeration produces a decoding matrix for every reasonable result that can be obtained from the encoding scheme.

958 A decoding matrix similar to that provided in Table 4 can be constructed for any encoding scheme described in this disclosure. Plausible results for degeneracy are then identified, and at least one potential analyte code is eliminated from the coding scheme to reduce or eliminate the degeneracy. Methods of eliminating degeneracy may be performed using software on a computer-readable medium, or using a hardware

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configuration (eg, a microchip) that performs the method.

[0119]

966 Although degeneracy can be reduced or eliminated by the methods described above, and elsewhere in this disclosure, this disclosure also provides encoding schemes that are non-degenerate by design.

968 For example, after eliminating the degeneracy of the encoding scheme described in Table 3, the encoding scheme can be expanded indefinitely in a non-degenerate manner, where the non-degeneracy is by design (see, e.g., the encoding scheme exemplified in Table 6, below described more fully). Similarly, the present disclosure provides an encoding scheme that is completely non-degenerate by design, and thus does not require any degeneracy reduction or elimination (see, eg, the encoding scheme exemplified in Table 8, described more fully below). Therefore, the encoding schemes provided by this disclosure may have reduced or eliminated degeneracy by design, or be non-degenerate by design.

[0120]

978 D.

979 An encoding method that uses more than one color and more than one intensity

[0121]

983 The encoding methods described above (eg, Tables 3-5) can be further extended by allowing analytes to be encoded by an intensity greater than 1.

985 For example, the coding scheme provided in Table 5 encodes each analyte that is coded by a fluorescence intensity of 1 or 0. Allowing higher signal intensity values 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 the control color in order to maintain the analyte counting capability of the control color.

[0122]

993 Table 6 shows the first three layers of an exemplary encoding scheme utilizing four colors and multiple intensities.

995 Layer 1 of Table 6 is a reproduction of Table 3, showing the coding of seven analytes and one control using four colors. As described above, any two of the potential analytes D, E, and F can be eliminated from the encoding scheme to produce a non-degenerate encoding scheme. Level 1 of Table 6 indicates that potential analytes D (1011) and F (1110) have been eliminated from the coding scheme to eliminate degeneracy. Therefore, as described above, the coding scheme shown in Level 1 of Table 6 is able to determine the presence of five analytes and one control using four colors.

[0123]

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1004 The coding scheme of Layer 1 of Table 6 can be extended to the second layer (Layer 2) by allowing the intensity of any color to increase.

1006 As described above, in order to maintain the sequence counting capability of the control, the intensity of the control color can be maintained at 1. Increasing the intensity of any of the remaining three colors will produce the codes 100Y, 10Y0, 1Y00, 10YY, 1Y0Y, 1YY0 and 1YYY, where Y>1. The minimum value of Y used to encode the new layer is equal to the cumulative maximum value from the existing layer plus one. As described below, values greater than 1 can also be used to maximize the difference between intensities.

[0124]

1014 Therefore, in this context, the term "layer" is generally used to describe a group of codes that take full advantage of the encoding capabilities provided by a specific number of first values (eg, intensity) and second values (eg, color) without degeneracy. .

1017 For example, Layer 1 of Table 6 takes full advantage of the encoding capabilities provided by four colors with no degeneracy and at most 1 intensity per color. As shown in Table 6, Tier 1, this would result in six coded analytes including controls. Introducing a second layer, as mentioned above, the minimum value of Y can be increased to be equal to the cumulative maximum result from the existing layer plus one (or more than one). In the example provided in Table 6, the intensity of the blue (control) color was kept at 1 to preserve the sequence counting capabilities of this color. Therefore, layer 2 includes five non-degenerate coding possibilities by increasing the intensity in the green, yellow and red channels to equal the cumulative maximum result in each of these channels according to layer 1 plus one get. All possibilities for these codes of layer 2 can then be enumerated, and the codes that cause degeneracy can be eliminated, or layer 2 can be made by design, using the information used to eliminate the corresponding codes from layer 1. Non-degenerate. Greater coding capacity can then be achieved by adding a third layer, or more layers, built according to a similar approach. The encoding scheme can have an unlimited number of layers, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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 layers.

[0125]

1034 More specifically, referring to Table 6, analyte H in layer 2 is encoded by 1004.

1035 The value of the control color remains at 1. The value of the red color is equal to 1 plus 1, or 4, based on the cumulative maximum result from the previous layer (3). Likewise, analytes I and J are coded by 1030 and 1400 respectively. The combination of these codes is used to encode the remaining four analytes (K-N), as done in layer 1 for analytes D-G. This completes layer 2. At level 2, the content of potential analytes K (1034) and M (1430) leads to degeneracy. Therefore, these analytes have been eliminated from the coding scheme to eliminate degeneracy.

[0126]

1044 Continuing with reference to Table 6, the third layer (Layer 3) is constructed using the same principles described above.

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1046 Analyte O is coded with 1-0-0-16. The value of the control color remains 1. The value of the red color is equal to 1 plus 1, or 16, based on the cumulative maximum result from the previous layer (15). Likewise, analytes P and Q are encoded by 1-0-9-0 and 1-16-0-0 respectively. A combination of these codes is used to encode the remaining four analytes (R-U), as done in layer 1 for analytes D-G and in layer 2 for analytes K-N. At level 3, the content of potential analytes R(1-0-9-16) and T(1-16-9-0) leads to degeneracy. Therefore, these analytes have been eliminated from the coding scheme to eliminate degeneracy.

[0127]

1055 The three-tier coding scheme shown in Table 6 shows the coding of 15 analytes and one control using four colors.

1057 This encoding scheme can be expanded indefinitely by adding more intensities to create additional layers and/or adding more colors to create additional encoding capabilities within layers. As described in this disclosure, methods to reduce or eliminate degeneracy can be used when adding each intensity and/or color to reduce or eliminate the results of degeneracy.

[0128]

1064 More generally, the encoding scheme depicted in Table 6 is a non-degenerate, infinite extension of the encoding scheme depicted in Tables 3-5.

1066 The maximum intensity measured cumulatively in the first layer is 6. The maximum intensity measured cumulatively in the second layer is 15. The maximum intensity measured cumulatively at level 3 is 63, and so on. Given the maximum cumulative intensity value (F), the maximum number of layers (T) available in this coding scheme is $T=\log4(F+1)$. The coding scheme depicted in Table 6 provides five (P) non-degenerate codes per layer. Therefore, the maximum number of codes $M=5*\text{Log}4(F+1)$, or $M=P*T$, where P is the number of non-degenerate codes per level and T is the number of levels. For example, given F=63, for 5 non-degenerate codes per layer, the maximum number of codes (ie, analytes) is 15. This formula does not include code 1000, which is retained as a positive control in Table 6. To include controls in the total number of analytes, one can simply add one, providing the formula $M=(P*T)+1$.

[0129]

1078 The methods provided in this disclosure can be used to infinitely scale up this encoding scheme.

1079 For example, by utilizing different combinations of intensity (i.e., first value) and color (i.e., second value), one can change the number of non-degenerate codes per layer (P) and the number of layers (T), encoding any number (M) of analytes. For example, the number of non-degenerate codes at each level 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 layers can 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.

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[0130]

1090 Table 6.

1091 Code 15 analytes and 1 control using four colors and multiple intensities.

[0133]

1095 The right-hand side of Figure 1 shows an illustrative example of the method described above.

1096 More specifically, the right-hand side of Figure 1 shows a coding method in which each of 9 or more analytes (Sequence 1 - Sequence 9, etc.) is represented by at least two colors, and within each color Intensity changes are encoded.

1099 Colors represent fluorophores attached to oligonucleotide probes that also include quenchers.

1100 Systems can be designed such that each probe is labeled with a single fluorophore, or each probe is labeled with more than one fluorophore. For example, the code for analyte H is 1004 in Table 6. An intensity of 4 in the red channel can be achieved by using H-specific probes that include 4 red fluorophores, or by using 4 H-specific probes that each include a single red fluorophore. Of course, any combination of probes and fluorophores that yields a result of 4 in the red channel is equally suitable, such as 2 probes each containing 2 red fluorophores, and 1 probe containing 1 red fluorophore. and 1 probe containing 3 red fluorophores, or just 1 probe containing 1 red fluorophore, but present in the reaction mixture at 4x the amount.

[0134]

1110 The encoding scheme depicted on the right-hand side of Figure 1 may be as shown in Table 7.

1111 The analysis result shown on the right hand side of Figure 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 sequence 1, sequence 3, sequence 4, and sequence 5.

[0135]

1118 Table 7.

1119 The right-hand side of Figure 1 illustrates the encoding scheme.

[0138]

1123 E.

1124 Use one color and one intensity per analyte, but use different encoding methods for intensities within analytes

[0139]

1129 In some of the methods presented here, each analyte is encoded by a single combination of color and

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intensity.

1131 For example, in a four-color system, the first four analytes could be coded by 1000, 2000, 4000, and 8000.

1132 The next four analytes can be coded by 0100, 0200, 0400, and 0800.

1133 Analytes 9-12 and 13-16 will be distributed similarly as shown in Table 8.

[0140]

1137 Like the encoding method described in Table 6, this encoding scheme is limited by the bandwidth of the instrument used by the construction to be theoretically infinite, non-degenerate, and only used by the measurement signal.

1140 However, this encoding scheme is able to quantify more analytes per unit bandwidth than the encoding method described in Table 6. The reason for this is that the available multiplexing of the signal is used with maximum efficiency, since each intensity level is utilized in the encoding (i.e. there are no gaps in the color spectrum; see description of the color spectrum below). Table 8 shows one embodiment of this method, illustrating a four-layer encoding based on four colors and intensities of 1, 2, 4 and 8. The coding scheme is non-degenerate, and if all 16 analytes are present, the result is 15-15-15-15. In terms of bandwidth utilization, this encoding method is more efficient than the encoding method given above. However, this method does not have the proofreading capabilities of the first solution because all results decoded into reasonable outputs in the first solution have gaps in the color spectrum.

[0141]

1152 Table 8.

1153 An example of an encoding method that uses one color and one intensity for each analyte, but uses different intensities across analytes.

[0144]

1158 The methods shown above, and exemplified in Table 8, can be extended by introducing additional colors and/or intensities.

1160 For example, you can use 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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.

1163 Additional intensity levels are also available, such as 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, etc.

1165 The method is generalizable and can be used to encode an unlimited number of analytes. Each analyte is represented by a code in a single color, where the value of the code in that single color is equal to the sum of all previous values plus one. For example, if the first code in a particular color contains 1, subsequent codes are 2, 4, 8, 16, 32, 64, 128, and so on. Other progressions are possible but would not be considered efficient based on bandwidth usage. However, one of ordinary skill in the art will recognize that when one wishes to maximize the spacing between values, less efficient bandwidth usage may be desired. Similarly, the value 1 may be excluded from the encoding scheme, for example, to maximize the difference in intensity between

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the first encoded value and the noise of the instrument. For example, in some cases, the coding scheme may start with a value of 2, which would provide the analyte code with a progression of 2, 4, 8, 16, 32, 64, 128, etc., while the progression of the cumulative result would It's 2, 4, 6, 8, 10, 12, 14, 16, etc. This approach creates uniform gaps in the possible cumulative results, allows for higher tolerance to noise than analyte code levels of 1, 2, 4, 8, 16, 32, etc., and This corresponds to the cumulative result series of 1, 2, 3, 4, 5, 6, 7, 8, etc. In this example, the increased robustness in measurements comes at the expense of a reduced number of codes available in the fixed bandwidth of multiplexing. For example, if only 63 multipath states can be reliably measured, then an encoding scheme starting at 1 provides 7 codes per color, while an encoding scheme starting at 2 provides 6 codes per color. If 4 colors are available, the former will provide 28 codes, while the latter will provide 24 codes. In summary, both starting signal intensity and magnitude (i.e., the difference between intensity values) can be measured to maximize the resolution of the instrument's noise and to maximize the difference between intensity values, thereby enhancing discrimination ability between different experimental results.

[0145]

1188 The encoding scheme illustrated in Table 8 is non-degenerate by design.

1189 Although the coding scheme illustrated in Table 8 uses both intensity and color to code each of the 16 analytes, it is also possible to code each analyte by simply utilizing intensity. For example, given the 16 analytes (A-P) provided in Table 8, a single color coding scheme encoding all 16 analytes could be assigned 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. This encoding scheme is non-degenerate by design, and the cumulative intensity results can be unambiguously decoded to indicate the presence or absence of analytes A-P. This encoding scheme is capable of detecting the presence or absence of M analytes, where $M=Log2(F+1)$ and F is the maximum cumulative value of signal (eg, signal intensity). By adding a second component to this signal (such as color, as depicted in Table 8), the capacity of this encoding scheme can be increased to $M=C*Log2(F+1)$, where C is used in the encoding scheme number of colors.

[0146]

1202 Any suitable value may be used for C or F, as described throughout this specification.

1203 For example, C can 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+1 can be at least about 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, 131072, 262144, 524288, 1048576, etc. As described elsewhere in this disclosure, the initial starting value of F and the steps in its series can also vary.

[0147]

1211 III.

1212 Analyte

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[0148]

1216 The analyte may be any suitable analyte analyzed using the methods and compositions of the invention, wherein the analyte is capable of interacting with a reagent to produce a measurable signal having at least two components.

1219 Analytes can be naturally occurring or synthetic. Analytes may be present in samples obtained using any method known in the art. In some cases, the sample may be processed before it is used for analysis of the analyte. The methods and compositions presented in this disclosure can be used in solution phase assays without the need for particles (eg, beads) or solid supports.

[0149]

1226 In some cases, the analyte can be a polynucleotide, such as DNA, RNA, peptide nucleic acid, and any hybrid thereof, wherein the polynucleotide includes any combination of deoxyribonucleotides and/or ribonucleotides.

1229 Polynucleotides may be single-stranded or double-stranded, or contain both double-stranded or single-stranded sequence portions. Polynucleotides may comprise any combination of nucleotides or bases, including, for example, uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine, isoguanidine Purine and any of its nucleotide derivatives. As used herein, the term "nucleotide" may include nucleotides and nucleosides, as well as nucleosides and nucleotide analogs, and modified nucleotides, including both synthetic and naturally occurring species. . The polynucleotide may be any suitable polynucleotide for which one or more reagents (or probes) as described herein can be generated, 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 (small Cajal body-specific RNA, scaRNA), microRNA (miRNA), double-stranded (dsRNA), ribozyme, riboswitch (riboswitch) or viral RNA. The polynucleotide may be contained in any suitable vector, such as a plasmid, cosmid, fragment, chromosome, or genome.

[0150]

1245 Genomic DNA can be obtained from naturally occurring or genetically modified organisms, or from artificially or synthetically produced genomes.

1247 Individual analytes, including genomic DNA, can be obtained from any source and using any method known in the art. For example, genomic DNA can be isolated with or without amplification. Amplification may include PCR amplification, multiple displacement amplification (MDA), rolling circle amplification, and other amplification methods. Genomic DNA can also be obtained by cloning or recombination methods, such as those involving plasmids and artificial chromosomes or other conventional methods (see Sambrook and Russell, Molecular Cloning: A Laboratory Manual., supra). Polynucleotides can be isolated using other methods known in the art, for example as disclosed in Genome Analysis: A Laboratory Manual Series (Volume I-IV) or Molecular Cloning: A Laboratory Manual. If the isolated polynucleotide is mRNA, it can be reverse transcribed to cDNA using conventional techniques, such as described in Sambrook and

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Russell, Molecular Cloning: A Laboratory Manual, supra.

[0151]

1260 The analyte can be a protein, polypeptide, lipid, carbohydrate, sugar, small molecule, or any other suitable molecule that can be detected by the methods and compositions provided herein.

1262 Analytes can be enzymes or other proteins. The analyte may be a drug or metabolite (eg, an anticancer drug, a chemotherapeutic drug, an antiviral drug, an antibiotic drug, or a biologic). The analyte can be any molecule such as a cofactor, receptor, receptor ligand, hormone, cytokine, blood factor, antigen, steroid, or antibody.

[0152]

1269 The analyte can be any molecule from any pathogen, such as a virus, bacteria, parasite, fungus, or prion (eg, PrPSc).

1271 Examples of viruses include those from the families Adenoviridae, Flaviviridae, Hepadnaviridae, Herpesviridae, Orthomyxoviridae, Papovaviridae, Paramyxoviridae, Picornaviridae, Polyviridae, Oncoviridae, Retroviridae, Rhabdoviridae, and Togaviridae. Specific examples of viruses include adenovirus, astrovirus, bocavirus, BK virus, coxsackie virus, cytomegalovirus, dengue virus, Ebola virus, enterovirus, Epstein-Barr virus, feline leukemia virus, hepatitis virus, A Hepatitis 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 1 Type, HIV type 2, human papillomavirus (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 Type, 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, parvovirus, polio virus, rabies virus, respiratory syncytial virus, retrovirus, rhinovirus, rotavirus coronavirus, rubella virus, variola virus, vaccinia virus, West Nile virus and yellow fever virus.

[0153]

1289 Examples of bacteria include those from the following genera: Bordetella, Borrelia, Brucella, Campylobacter, Chlamydia, Clostridium Clostridium, Corynebacterium, Enterococcus, Escherichia, Francisella, Haemophilus, Helicobacter), Legionella, Leptospira, Listeria, Mycobacterium, Mycoplasma, Neisseria, Pseudomonas Pseudomonas, Rickettsia, Salmonella, Shigella, Staphylococcus, Streptococcus, Treponema), Vibrio and Yersinia.

1294 Specific examples of bacteria include Bordetella parapertussis, Bordetella pertussis, Borrelia burgdorferi, Brucella abortus, Brucella canis (Brucella canis), Brucella melitensis, Brucella suis, Campylobacter jejuni, Chlamydia pneumoniae, Chlamydia psittaci, Chlamydia trachomatis (Chlamydia trachomatix), Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium tetani,

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Corynebacterium diphtheriae, Enterococcus faecalis (Enterococcus faecalis), Enterococcus faecium, Escherichia coli, Francisella tularensis, Haemophilus influenzae, Helicobacter pylori, Legionella pneumophila (Legionella pneumophila), Leptospira interrogans, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Mycobacterium ulcerans, Mycoplasma pneumoniae, Neisseria gonorrhoeae, Neisseria meningitidis, Pseudomonas aeruginosa, Rickettsia rickettsii, choleraesuis Salmonella choleraesuis, Salmonella dublin, Salmonella enteritidis, Salmonella typhi, Salmonella typhimurium, Shigella sonnei, Staphylococcus aureus (Staphylococcus aureus), Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus agalactiae, Streptococcus pneumoniae, Streptococcus pyogenes, Treponema pallidum (Treponema pallidum), Vibrio cholerae, Yersinia pestis and Yersinia enterocolitica.

[0154]

1310 Examples of parasites include those from the genera Acanthamoeba, Babesia, Balamuthia, Balantidium, Blastocystis Blastocystis, Cryptosporidium, Dientamoeba, Entamoeba, Giardia, Isospora, Leishmania Leishmania, Naegleria, Pediculus, Plasmodium, Rhinosporidium, Sarcocystis, Schistosoma, Toxoplasma, Trichomonas and Trypanosoma.

1314 Specific examples of parasites include Babesia divergens, Babesia bigemina, Babesiaequi, Babesia microti, Babesia ducani, Balamuthia mandrillaris, Balantidium coli, Dientamoeba fragilis, Entamoeba histolytica, Giardialamblia, Isospora belli, Naegleria fowleri, Pediculus humanus, Plasmodium falciparum, Plasmodium knowlesi, malaria malaria Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, Rhinosporidium seeberi, Sarcocystis bovihominis, Sarcocystis suihominis, Schistosoma mansoni, Toxoplasma gondii, Trichomonas vaginalis, Trypanosoma brucei, and Trypanosoma cruzi.

[0155]

1323 Examples of fungi include those from the genera Apophysomyces, Aspergillus, Blastomyces, Candida, Cladosporium, Coccidioides (Coccidioides), Cryptococcus, Exserohilum, Fusarium, Histoplasma, Pichia, Pneumocystis, Saccharomyces, Sporothrix, Stachybotrys and Trichophyton.

1326 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, Schenck Sporothrix schenckii, Stachybotrys chartarum and Trichophyton mentagrophytes.

[0156]

1333 In some cases, the methods provided in this disclosure can be used to detect any of the analytes described above, or elsewhere in the specification.

1335 In some cases, the methods provided in this disclosure can be used to detect panels of analytes described above, or elsewhere in the specification. For example, the combination may include 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, An analyte from the group consisting of 48, 49, 50, 60, 70, 80, 90, 100 or more of the analytes described above or elsewhere in the specification.

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[0157]

1343 Analytes can be obtained from any suitable location, including from organisms, intact cells, cell preparations, and cell-free compositions from any organism, tissue, cell, or environment.

1345 Analytes may be obtained from environmental samples, biopsies, aspirates, formalin-fixed embedded tissue, air, agricultural samples, soil samples, petroleum samples, water samples, or dust samples. In some cases, analytes can be obtained from body fluids, which may include blood, urine, feces, serum, lymph fluid, saliva, mucosal secretions, sweat, central nervous system fluids, vaginal fluids, or semen. Analytes can also be obtained from manufactured products such as cosmetics, foods, personal care products, etc. Analytes can be the product of experimental procedures, including recombinant cloning, polynucleotide amplification, polymerase chain reaction (PCR) amplification, purification methods (such as purification of genomic DNA or RNA), and synthesis reactions.

[0158]

1356 In each multiplex assay, more than one type of analyte can be detected.

1357 For example, polynucleotides, proteins, polypeptides, lipids, carbohydrates, sugars, small molecules, or any other suitable molecules can be detected simultaneously in the same multiplex assay with appropriate reagents. Any combination of analytes can be detected at the same time.

[0159]

1363 Detection of analytes can be useful for any suitable application, including scientific, clinical, diagnostic, prognostic, forensic and monitoring applications.

1365 Typical applications include detection of genetic diseases, identification of genetic fingerprints, diagnosis of infectious diseases, gene cloning, paternity testing, criminal identification, phylogeny, bio-anti-terrorism, environmental monitoring, and DNA computing. For example, an analyte may be indicative of a disease or condition. Analytes can be used to make treatment decisions, or to assess disease status. The presence of the analyte may be indicative of infection with a specific pathogen or any other disease such as cancer, autoimmune disease, cardiopulmonary disease, liver disease, gastrointestinal disease, etc. The methods presented here can therefore be used to make a diagnosis and make clinical decisions based on that diagnosis. For example, results indicating the presence of bacterial polynucleotides in a sample collected from a subject may result in treatment of the subject with antibiotics.

[0160]

1377 In some cases, the methods and compositions of the invention can 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.

1381 In some cases, the methods and compositions of the invention can be used to detect 7-50, 8-40, 9-30, 10-20,

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10-15, 8-12, or 7-12 analytes.

[0161]

1386 In some cases, the present disclosure provides the ability to unambiguously detect any combination of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, in the presence or absence in a single sample volume. 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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, The presence of each of 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes or Absence is determined without the need for immobilization, separation, mass spectrometry, or melting curve analysis.

1392 In some cases, the present disclosure provides the ability to unambiguously detect at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 in any combination of presence or absence in a single sample volume ,13,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37 ,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 The presence of each of , 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes or absence of assay without immobilization, separation, mass spectrometry, or melting curve analysis. In some cases, the present disclosure provides the ability to unambiguously detect less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, in any combination of presence or absence in a single sample volume. 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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, Determination of the presence or absence of 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes No immobilization, separation, mass spectrometry, or melting curve analysis is required.

[0162]

1407 IV.

1408 Signal

[0163]

1412 The methods presented in this disclosure can be used with any quantifiable signal.

1413 In some cases, the present disclosure provides encoding schemes and methods to non-degenerately encode an unlimited number of objects using a single component of a signal (eg, intensity). For example, as described above and in Example 3, the encoding scheme may rely on multiplexing of signal strength without regard to color. Although fluorescent probes have been used to illustrate this principle, as described elsewhere in this disclosure, the encoding scheme is equally suitable for any other method that provides a quantifiable signal, including electrochemical signals and chemical signals. Glow signal.

[0164]

1422 The methods proposed by this disclosure may also make use of measurements of a signal in at least two dimensions, also referred to as measurements of at least two components of a signal.

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1424 For example, utilizing at least two components of a signal (e.g., color and intensity) allows for more differences per unit of signal intensity bandwidth than the encoding scheme described in the paragraph above that relies on signal intensity to distinguish analytes. code. When at least two components of the signal are utilized, cumulative measurements of at least two components of a single sample volume can be obtained. For example, in the encoding scheme described in Table 5, the presence of each analyte results in a specific intensity (one component of the signal) in each of 4 colors (the second component of the signal). The combination of these constituent signals results in a cumulative signal that can be measured by measuring the intensity at each wavelength or range of wavelengths. The cumulative measurements can then be converted into a determination of the presence or absence of the analyte using a corresponding encoding scheme or decoding matrix.

[0165]

1437 In some cases, a quantifiable signal includes a waveform having both frequency (wavelength) and amplitude (intensity).

1439 The signal may be an electromagnetic signal. The 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, the electromagnetic signal may be a fluorescent signal, such as a fluorescence emission spectrum that may be characterized in terms of wavelength and intensity.

[0166]

1446 In certain portions of this disclosure, signals are described and exemplified in terms of fluorescent signals.

1447 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 fluorescent signals also apply to other signals. For example, like fluorescent signals, any of the electromagnetic signals described above can be characterized in terms of wavelength and intensity. The wavelength of a fluorescent signal can also be described in terms of color. Color may be determined based on measuring intensity at a specific wavelength or range of wavelengths, for example, by determining the intensity distribution of fluorescence at different wavelengths, and/or by utilizing a bandpass filter to determine the intensity of fluorescence within a specific wavelength range. Such bandpass filters are commonly used in a variety of laboratory instruments, including quantitative PCR machines. Intensity can be measured with a photodetector. Many wavelengths can be called "channels."

[0167]

1459 In some cases, the methods provided in this disclosure can be used with any signal where the cumulative signal measures the same color, frequency, absorption band, etc. as the constituent signals.

1461 However, the accumulation signal need not be digital or scale linearly with the number and strength of the constituent signals. For example, if the physical principle of measurement is absorption, then due to the exponential nature of Beer-Lambert's law, the cumulative attenuation is the product of the constituent attenuations while the constituent concentrations are additive. The logarithm of the cumulative attenuation will then scale linearly with the constituent concentration at each absorption band (corresponding to color if

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fluorescence detection is used). Therefore, the method of the present invention is applicable. The method of the present invention can also be used for chemiluminescent signals and electrochemical signals.

[0168]

1471 The number of signals, and the number of dimensions or components measured, may also be expanded beyond that shown in the exemplary embodiments of the present invention, resulting in an expansion of multiplexing capabilities.

1474 Exemplary embodiments provided in this disclosure utilize encoding schemes constructed using fluorescent signals having 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 fluorescent signals with electrochemical signals or FRET signals (fluorescence resonance energy transfer).

[0169]

1483 In some cases, more than two components of the signal can be measured.

1484 For example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, 20 or more components of the 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 the signal may be measured. At least 2, but less than 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, or 20 components of the 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 the signal may be measured. These additional components may include kinetic components such as the rate of signal decay and the rate of photobleaching.

[0170]

1493 If fluorescent signals are employed, the number of analytes that can be encoded can be further expanded by utilizing additional fluorophores.

1495 For example, you can use 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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. 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. In some cases, less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 may be used ,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44,45,46,47 , 48, 49, or 50 fluorophores. 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 can be used , or 10-20 fluorophores.

[0171]

1505 Typically, the number of analytes that can be encoded can be further expanded by utilizing an additional first value (eg, intensity) from a value or range of values of the first component of the signal.

1507 For example, you can use 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26,

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27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more first values from the value or range of values of the first component of the signal. 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 from a value or range of values of the first component of the signal. In some cases, less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 may be used ,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44,45,46 , 47, 48, 49, or 50 first values from the value or range of values of the first component of the signal. 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 can be used , 10-15, or 10-20 from the value or range of values of the first component of the signal.

[0172]

1520 In examples where intensity is a component of the signal being quantified, such as when utilizing a fluorescent signal, the presence of the analyte may be encoded using multiple intensities or intensity ranges.

1522 For example, the encoding 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 intensity ranges. Encoding schemes may utilize at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 22, 23, 24
,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44,45,46,47,48,49 , 50, or more intensities or intensity ranges. Encoding schemes can utilize less 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 intensity ranges. In some cases, encoding schemes 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 intensity ranges.

[0173]

1535 The number of analytes that can be encoded can be further expanded by utilizing an additional second value (eg, wavelength) from the value or range of values of the second component of the signal.

1537 For example, you can use 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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 from the value or range of values of the second component of the signal. 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 from a value or range of values of the second component of the signal. In some cases, less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 may be used ,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44,45,46 , 47, 48, 49, 50, or more second values from a value or range of values of the second component of the signal. 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 can be used , 10-15, or 10-20 second values from the value or range of values of the second component of the signal.

[0174]

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1550 In instances where wavelength is a component of the signal being quantified, such as when utilizing a fluorescence signal, the presence of the analyte may be encoded using multiple wavelengths or wavelength ranges.

1553 For example, you can use 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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. 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. In some cases, less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 may be used ,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44,45,46 , 47, 48, 49, or 50 wavelengths or wavelength ranges. 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 can be used , 10-15, or 10-20 wavelength or range of wavelengths.

[0175]

1565 In some cases, when degeneracy is eliminated, the methods of the present invention are capable of detecting 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, The presence or absence of 44, 45, 46, 47, 48, 49, 50, or more analytes when each reagent used to generate a signal in the volume produces only one second value (e.g., each reagent , when only one wavelength of light is emitted).

[0176]

1573 In other cases, when degeneracy is eliminated, the method of the present invention is capable of detecting 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, The presence or absence of 45, 46, 47, 48, 49, 50, or more analytes, using a total of four second values in the volume (e.g., a total of four wavelengths or ranges of wavelengths that may Implemented by using four spectrally resolvable fluorophores).

[0177]

1581 As described throughout the specification, the assays provided herein utilize cumulative measurements of samples.

1583 The cumulative measurement may be, for example, a single measurement of an intensity value, or a measurement of intensity values at one or more wavelengths or ranges of wavelengths.

1585 Multiple cumulative measurement results can be obtained.

1586 For example, intensity can be in the range 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, or more wavelengths or wavelength range measurements. Intensity can be 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 wavelength range measurements. Intensity can be in less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23

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,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44,45,46,47,48 , 49, or 50 wavelengths or wavelength range measurements.

[0178]

1597 More generally, cumulative measurements can be obtained with respect to any quantifiable component of the signal, and with respect to any quantifiable component of the signal at another quantifiable component of the signal.

1600 For example, at least one first component of the signal may be 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 component measurements of the signal. The at least one first component of the signal may be 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, Second component measurement of 46, 47, 48, 49, 50, or more signals. At least one first component of the signal may be present in 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 component measurements of the signal.

[0179]

1612 As will be apparent from this disclosure, each analyte to be detected may be encoded as a suitable component using any number of signals, or as a code for any number of signals.

1614 For example, each analyte to be detected could be coded 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 components of the signal or in the signal. 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 or in the signal. In some cases, each analyte to be detected may be encoded in 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 components of or in the signal. 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 can be used A component or signal of , 5-10, 10-15, or 10-20 encodes each analyte. In the encoding scheme each analyte may be encoded by the same number of components of the signal, or signals, or by a different number of components of the signal, or signals.

[0180]

1629 A.

1630 Probes, primers, fluorophores, and quenchers

[0181]

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1634 Some methods provided in this disclosure utilize reagents that generate a signal in the presence of an analyte.

1635 Any suitable reagent can be used in the present invention. Typically, the 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 called probes. The probe may be a hybridization probe. The hybridization probe can be an oligonucleotide probe linked to a fluorophore and a quencher (eg, TAQMAN probe).

[0182]

1642 Methods of the invention may use one or more reagents or probes to detect the presence or absence of each analyte.

1644 For example, you can use 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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 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 to detect the presence or absence of each analyte. In some cases, less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 may be used ,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44,45,46,47 , 48, 49, 50 probes 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.

[0183]

1656 In some cases, samples were associated 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 are contacted to detect the presence or absence of all analytes.

1659 In some cases, the sample is associated 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 probe contacts are made to detect the presence or absence of all analytes. In some cases, samples were associated with less 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 probe contacts to detect the presence or absence of all analytes. In some cases, the number of probes to which the sample is exposed 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.

[0184]

1669 As described above, oligonucleotide probes linked to fluorophores and quenchers can be used to detect the presence of analytes in polynucleotide amplification assays.

1671 As long as the quencher and the fluorophore are in proximity, the quencher quenches the fluorescence emitted by the fluorophore when excited by the light source. The sequence of the oligonucleotide probe is designed to be complementary to the polynucleotide sequence present in the analyte and, therefore, capable of hybridizing to the polynucleotide sequence present in the analyte. Hybridization of oligonucleotide probes can be performed in a nucleic acid amplification reaction (eg, polymerase chain reaction) involving

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primers. After extension of the primer by DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and quencher into the medium. The proximity between the fluorophore and the quencher is destroyed, and the signal from the fluorophore is no longer quenched. Therefore, 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 the analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be generated.

[0185]

1685 Oligonucleotide probes can have one or more fluorophores and quenchers per probe.

1686 For example, in some embodiments, oligonucleotide probes may include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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. Oligonucleotide probes may include 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. Oligonucleotide probes may include less 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.

[0186]

1697 Oligonucleotide probes may include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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.

1700 Oligonucleotide probes may include 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. Oligonucleotide probes may include less 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.

[0187]

1708 Ligation of the probe and quencher to the probe can be performed in the same reaction, or in a series of reactions.

1710 A series of reactions can be performed to label the probe with at least one fluorophore, and the reaction products can be mixed to produce a mixture of probes with different fluorophores.

[0188]

1715 If the oligonucleotide probe includes two or more fluorophores, these fluorophores can be arranged to allow (fluorescence) resonance energy transfer (FRET) to occur between the

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fluorophores.

1719 Simply put, FRET is a mechanism of energy transfer between fluorophores. The use of FRET-based probes allows a single excitation source to produce two distinct fluorescence emission signals via excitation of a single fluorophore. For example, the methods described herein can use paired probes, where one probe in the pair is attached to a fluorophore and a quencher, and the second probe is attached to both fluorophores (close enough for FRET to occur) and Quencher. Any combination of fluorophores and quenchers provided for FRET can be used. This approach doubles the number of fluorescent probes that can be used with an excitation source because a single excitation source can be used to generate two signals: one from a probe with one fluorophore and one from a probe with a fluorophore close enough for FRET Probes that occur with two fluorophores. For example, using the encoding method described in Table 8, the number of unambiguously detectable analytes can be increased from 16 to 32 by pairing each probe in Table 8 with a corresponding FRET probe.

[0189]

1733 Primers can be specific for a particular analyte and can amplify a region complementary to the probe.

1734 In some cases, the number of primer pairs used is equal to the number of probes. In other cases, the number of probes used may exceed the number of primer pairs used. In yet other cases, the number of primer pairs used may exceed the number of probes used. In some cases, the sample to be analyzed may be associated 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 primer pair contacts. In some cases, the sample to be analyzed may be associated 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, Paired contacts of 45, 46, 47, 48, 49, 50, or more primers. In some cases, the samples to be analyzed may be associated with less 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 primer pair contacts. In some cases, the number of primer pairs is 2-10, 3-15, 4-20, 3-10, 4-10, 5-10, 6-8, or 6-10.

[0190]

1749 Primers can amplify regions of the polynucleotide to which different numbers of hybridization probes hybridize.

1751 For example, at least one pair of primers can amplify genes with 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 regions complementary to hybridization probes. In some cases, all of said pairs of primers can amplify with 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 regions complementary to the hybridization probe.

[0191]

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1760 In one example, the first probe can be labeled with the fluorophore 5'-fluorescein amide (5'-FAM) and the quencher black hole quencher 1 (BHQ-1).

1762 The second probe can be labeled with 5'-FAM in close proximity to (eg, linked to) cyanine 5.5 (Cy5.5) and the quencher black hole quencher 3 (BHQ-3). Upon digestion of the nuclease activity probe via a polymerase, two fluorescent signals are generated from a single excitation wavelength (eg, 470 nm). The fluorophore from the first probe fluoresces at approximately 520 nm. The fluorophore on the second probe is subjected to FRET. The donor fluorophore FAM is excited by an excitation light source (eg, 470 nm) and transfers its energy to the acceptor fluorophore Cy5.5. The acceptor fluorophore emits at approximately 705nm. These fluorophores and quenchers are exemplary only. Any fluorophore capable of undergoing FRET, and any quencher that can quench fluorescence, is suitable for use in the present invention. Methods for producing FRET-based probes are described in Jothikumar et al., BioTechniques, 2009, pages 46(7):519-524.

[0192]

1775 In some embodiments, a single hybridization probe can be used for each analyte.

1776 To take advantage of multi-color coding (eg, as shown in Table 3), each probe can be labeled with multiple fluorophores in predetermined ratios. The ratio can be determined so that the 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 cheaper to deploy than approaches utilizing multiple probes. Furthermore, in the case of hybridization probes, it is easier to match one hybridization probe to the sequence of the analyte than multiple probes.

[0193]

1785 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 will work equally well with the invention described in this disclosure. good.

1788 For example, binding molecules specific for the analyte can be used as probes. Non-limiting exemplary binding molecules include antibodies that recognize an analyte and generate a signal in the presence of the analyte.

[0194]

1794 In embodiments of the invention utilizing fluorescent labels, any suitable fluorescent label may be used.

1795 Exemplary fluorescent labels suitable for use in the present invention include rhodamine, parametaminophen, fluorescein, thiofluorescein, aminofluorescein, carboxyfluorescein, chlorofluorescein, methylfluorescein, sulfonate fluorescein, aminorhodol, carboxyrhodol, chlororhodol, methylrhodol, sulforhodol), aminorhodamine, carboxyrhodamine, chlororhodamine, methylrhodamine, sulforhodamine, thiorhodamine, cyanine), indolecarbocyanine, 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,

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Nile red, Nile blue, cresyl violet, oxazine 170, acridine derivatives, diaminoacridine (proflavin), acridine orange, acridine yellow, arylmethine) derivatives, auramine, crystal violet, malachite green, tetrapyrrole derivatives, porphines, phthalocyanine pigments and bilirubin. Exemplary quenchers include black hole quenchers, such as BHQ-0, BHQ-1, BHQ-2, BHQ-3; ATTO quenchers, such as ATTO 540Q, ATTO 580Q, and ATTO 612Q; and the like.

[0195]

1810 Fluorophores that can be used with the present invention are not limited to any fluorophores described herein.

1812 For example, fluorophores with improved properties continue to be developed, and these fluorophores can be readily used with the methods provided by this disclosure. This improved fluorophore includes quantum dots that can 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 required, but many different signals can be quantified, for example, in terms of color and intensity. Furthermore, fluorophores with narrow emission spectra would be particularly useful for the methods described here, as such fluorophores can be included in multiplex assays with little or no overlap between their emission spectra, thus providing more "color" and significantly increase the overall number of coded analytes.

[0196]

1823 In some cases, one or more reagents are lyophilized.

1824 Any suitable reagent can be lyophilized. For example, probes, primers, enzymes, antibodies, or any other reagents used for detection can be lyophilized. In some cases, samples including analytes 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 cannot be reliably utilized. In one example, lyophilized reagents include any probe or analyte-specific reagent described in this disclosure or otherwise suitable for use in the present invention. In another example, the lyophilized reagents include PCR primers. In yet another example, lyophilized reagents include reagents suitable for performing PCR reactions.

[0197]

1835 V.

1836 Analytical techniques and instruments

[0198]

1840 The methods provided in this article are suitable for use with various detection methods.

1841 For example, applicable methods use analytical techniques that measure the wavelength and intensity of a fluorescent signal. This can be accomplished by measuring the intensity of the signal across the entire spectrum of wavelengths, or by using a bandpass filter that limits light at certain wavelengths, allowing only

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certain wavelengths of light to reach the photodetector. Many real-time PCR and quantitative PCR instruments include an excitation light source and a band-pass filter capable of detecting fluorescent signals in four colors (eg, blue, green, yellow, and red). Therefore, the method of the present invention can be easily applied using instruments widely used in the art. Importantly, the methods and compositions provided herein can be used to detect multiple analytes by obtaining cumulative measurements of a single solution. There is no need to separate. The present invention does not require the use of beads or solid phases. Of course, one of ordinary skill in the art will understand that the present invention can be used in conjunction with separation, beads, or solid phases if desired.

[0199]

1855 Figure 5 shows two examples of configurations of instruments that can be used to obtain cumulative measurements useful for the method of the invention.

1857 Referring to Figure 5, an instrument is shown at 501 with a detector (eg, a fluorometer) configured to detect both wavelength and intensity. Referring to 501 , at least one excitation light source 502 is directed into a chamber 503 containing an analyte and a reagent that generates a signal in the presence of analyte 504 . As described elsewhere, the analyte may be a nucleic acid and the signal-generating reagent may be a hybridization probe including a fluorophore and a quencher. If the analyte is present, an emission signal 505 is generated. In the configuration shown at 501, the wavelength and intensity of the emission signal are measured across the spectrum by a detector capable of producing a fluorescence emission spectrum 506.

[0200]

1867 Wavelength and intensity can also be determined using a combination of photodetectors and bandpass filters.

1869 This configuration is used in several thermal cyclers known in the art. Referring to Figure 5, an instrument with a bandpass filter and spot detector is depicted at 507. Referring to 507, at least one excitation light source 508 is directed into a chamber 509 containing an analyte and a reagent that generates a signal in the presence of analyte 510. As described elsewhere, the analyte may be a nucleic acid and the signal-generating reagent may be a hybridization probe including a fluorophore and a quencher. If the analyte is present, an emission signal 511 is generated. In the configuration shown at 507, bandpass filters 512, 513, and 514 are used to limit the passage of light to emit light within a specific wavelength range. For example, bandpass filter 512 may limit the passage of light to emit light in wavelength range 1. Bandpass filter 513 can limit the passage of light to emit light in wavelength range 2. Bandpass filter 514 may limit the passage of light to emit light within wavelength range 3. Any number of bandpass 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 in the present invention. Furthermore, the location of the bandpass filter in 507 is not meant to be limiting. In some cases, the emitted light may pass through more than one bandpass filter at any time. In other cases, the bandpass filters may be configured such that all emitted light passes through only one bandpass filter at a time. After passing through the bandpass filter, the filtered light 515, 516 and 517 is detected by the 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 filtered light 515, 516, and

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517 can then be quantified by photodetector 518.

[0201]

1890 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.

1893 Digital PCR methods (eg, droplet digital (BIORAD) and dynamic array (FLUIDIGM)) produce highly sensitive quantification of polynucleotide copy number. The methods presented here can be easily integrated into these systems, significantly expanding their working capabilities by allowing multiplexing in microdroplets or dynamic arrays.

[0202]

1900 Similarly, the methods described in this disclosure can be applied in real-time PCR assays.

1901 For example, as a PCR run is completed, real-time data can be fully recorded. The endpoint value can then be decoded to indicate the presence or absence of the analyte. The value of each cycle threshold (Ct) can be analyzed for each color by detecting the maximum value of the second derivative of the real-time curve for that color. Because a specific analyte is amplified, the fluorophore is released from its corresponding probe in the same proportion as the color diversity encoded by that sequence. So a code like 1100 will have the same Ct in blue and green, while a 1400 code will have a green Ct 2 cycles ahead of its blue Ct. This additional data enables determination of the identity and starting amount of each analyte.

[0203]

1911 The method described here can also be used directly on tissue samples.

1912 For example, a tissue sample can be obtained and wells can be created directly in the tissue sample using photolithography. Tissue samples can be fixed in advance to create wells. The tissue sample can then be analyzed by dispensing appropriate reagents into the wells in the tissue sample. The encoding and decoding methods described in this disclosure can be used for multiplexed detection of analytes etched into wells in tissue. Each well can correspond to a single cell or several cells, providing excellent spatial resolution when performing analyte analysis on different parts of the tissue. This method can be used to detect analytes in different regions of the same tissue.

[0204]

1922 In some cases, the instrument may be modified or constructed, for example, to provide additional excitation light sources at multiple wavelengths, and/or to provide additional bandpass filters or the ability to determine the complete spectrum.

1925 Inclusion of additional excitation sources will allow excitation of a greater number of various fluorophores. Including additional bandpass filters, or modifying or constructing the instrument to be able to determine the entire spectrum, allows detection of a wider variety of emissions from the fluorophore. These techniques

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can be used to increase the number of fluorophores that can be used in the methods described in this disclosure, and thus the number of analytes that can be detected simultaneously.

[0205]

1933 In some cases, methods described in this disclosure utilize end-point PCR methods.

1934 However, in some cases, results may be obtained before the endpoint. This may be advantageous, for example, when results are needed as quickly as possible. For example, one performs a calibration experiment in which different starting concentrations of the analyte are analyzed to determine the number of cycles at which the signal reaches saturation as a function of the starting amount of analyte. Using this data, a computer that monitors PCR reactions in real time can be programmed to search for saturation at the maximum number of cycles based on the limits of the limit of detection (LOD) of a particular system. If the analyte does not amplify by the maximum cycle number (eg, except for a possible positive control), then the result is negative for that analyte. If there is amplification of the analyte by the maximum cycle number, then the result is positive for that analyte, and the saturation intensity in each color can be used to decode the result using a coding scheme. In both cases, there is no need to run the PCR reaction beyond the number of cycles set by the LOD for the instrument. Although PCR methods have been used to illustrate this quantitative method, one of ordinary skill in the art will readily recognize that similar principles can be applied in any catalytic system where the starting amount of analyte limits the reaction rate, and the reaction Rate can be measured and reported by the evolution of signal strength over time.

[0206]

1951 VI.

1952 Compositions and kits

[0207]

1956 The present disclosure also provides compositions and kits for use with the methods described herein.

1957 The compositions can include any of the ingredients, reaction mixtures and/or intermediates described herein, and in any combination. For example, the present disclosure provides detection reagents for use with the methods provided herein. Any suitable detection reagent may be provided, including hybridization probes labeled with fluorophores and quenchers and primers as described elsewhere in this specification.

[0208]

1964 In some cases, the composition includes a reagent that detects at least seven analytes using four fluorophores.

1965 In some cases, the composition includes using four fluorophores to detect 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. In some cases, the composition includes using five fluorophores to detect 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 analyte reagents. In

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some cases, the composition includes using six fluorophores to detect 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 reagents for 50 analytes. In some cases, the composition includes using seven fluorophores to detect 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 Reagents for 50 analytes.

[0209]

1978 In some cases, the composition includes primers.

1979 The composition may include 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 primer pairs.

[0210]

1984 The invention also provides kits for carrying out the methods of the invention.

1985 Therefore, various kits are provided in suitable packaging. A kit 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 of a plurality of analytes. The kit may include a coding scheme, or decoding matrix, to assist the user in converting cumulative measurements into results indicating the presence or absence of each of a plurality of analytes. The kit may be a diagnostic kit, for example, a diagnostic kit suitable for the detection of any analyte including the analytes enumerated herein. Kits may contain any of the compositions provided in this disclosure, including those enumerated above.

[0211]

1995 Figure 6 shows a schematic diagram of an exemplary kit of the invention.

1996 Referring to Figure 6, a kit is shown that includes a cartridge 601 containing reagents 602 for detection of an analyte (eg, a polynucleotide probe including a fluorophore and a quencher), and reagents 602 for nucleic acids. Amplification primer 603. The kit may also contain any other reagents, such as reagents 604 suitable for performing an amplification reaction. In the embodiment depicted in 601, the reagents and primers for detection of the analyte are each contained in a single volume (eg, tube or bottle). However, these reagents may also be provided in separate volumes, as depicted by the box shown at 605. Box 605 contains seven separate volumes. Three volumes 606, 607 and 608 contain reagents (or mixtures of such reagents) for detection of analytes, and three volumes 609, 610 and 611 contain primers (or mixtures of primers). The kit may also contain reagents 604 suitable for performing amplification reactions.

[0212]

2008 Figure 6 is provided for illustration purposes only.

2009 Any number of reagents for detection of analytes (eg, probes), and any number of primers can be included in a single tube or bottle, making it suitable 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 may be included in a single bottle

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,19,20,21,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42,43 , 44, 45, 46, 47, 48, 49, 50, or more reagents for detection of analytes. In other cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, may be included in a single bottle or tube. 19, 20, 21, 22, 23, 24, 25, 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. In some cases, primers and probes may be provided in the same bottle or tube. For example, each primer pair can provide 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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. In other cases, each probe pair can provide 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 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. The kit can 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 separate bottles or tubes, or, for example, in the bottle or tube 604 that provides the reagents for the amplification reaction. Any reagents in the kit can be lyophilized as described elsewhere in this specification.

[0213]

2029 VII.

2030 Systems and software

[0214]

2034 The methods of the present disclosure may also be performed as part of a system.

2035 For example, one or more steps of the methods described herein may be performed by software embodied in a computer-readable medium. Software can be used to encode analytes, check degeneracy, reduce or eliminate degeneracy, and/or generate decoding matrices. Software can also be used to convert cumulative measurements made on an instrument (eg, a PCR machine) into information about the presence or absence of an analyte. For example, referring to Figure 4, computer 401 may be connected to an input device 403, a display 402, and a printer 404. The computer may perform one or more steps of any of the methods described in this disclosure, including, for example, the encoding method 410, the analysis method 420, and the decoding method 430. The computer may reduce or eliminate degeneracies, for example, as part of encoding method 410. Similarly, a computer can generate encoding schemes that are non-degenerate by design, such as those designed using mathematical iteration. Computers can also be connected to the network 405. The network may be a local area network and/or the Internet. A network can be used to perform the method of the invention on multiple computers in different locations. For example, one computer can perform encoding, another computer can perform measurement, and yet another computer can perform decoding. A computer can perform any or all of these methods. Networks may be used to transmit information obtained using the methods described in this disclosure throughout the world, such as across national borders.

[0215]

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2054 In one example, the software is embedded in the instrument that performs the measurements, such as a thermal cycler.

2056 In another example, the software is installed on a computer connected to the instrument performing the measurements, such as a computer connected to a thermal cycler. In another instance, the software is "in the cloud," that is, on another computer that can communicate with it over a network.

[0216]

2062 Each element described above can be connected to a controller, which communicates with each element and coordinates their operation.

2064 For example, the controller may initiate encoding of a series of analytes into color and intensity values. 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 can then automatically order probes corresponding to that code from the probe's manufacturer. Alternatively, the controller can operate an oligonucleotide synthesizer that can synthesize corresponding probes. After analyzing the sample on the instrument (which can also be automated), the controller can process the results of the analysis using a decoding matrix. The controller can then provide these results to the user.

[0217]

2074 VIII.

2075 Serve

[0218]

2079 The methods provided in this article can also be executed as services.

2080 For example, a service provider may obtain the identities of multiple analytes that a customer wishes to analyze. The service provider can then encode each analyte to be detected by any of the methods described herein and provide the customer with the appropriate reagents for the assay. The client can also perform measurements and provide the results to the service provider for decoding. The service provider can then provide the decoded results to the client. Customers can also encode analytes, generate probes, and/or decode results by interacting with software installed locally (at the customer's location) or remotely (eg, on a server reachable over a network). Exemplary customers include clinical laboratories, physicians, food and consumer product manufacturers, industrial manufacturers (e.g., oil companies), and others. The customer or party may be any suitable customer or party having a need or wish to use the methods, systems, compositions and kits of the invention.

[0219]

2093 Example

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[0220]

2097 Example 1: General Materials and Methods

[0221]

2101 1.

2102 General reagents

[0222]

2106 TE buffer, pH7 (LIFE TECHNOLOGIES, Carlsbad, CA); RNase-free ultrapure water (LIFE TECHNOLOGIES, Carlsbad, CA); Taq 5x master mix (FISHER SCIENTIFIC COMPANY, Tustin, CA).

[0223]

2111 2.

2112 DNA sequences, primers and probes

[0224]

2116 Five nucleic acids from clinically relevant organisms were selected for illustrative studies: (1) human immunodeficiency virus 1 (HIV-1); (2) Plasmodium falciparum (malaria); (3) herpes simplex virus 2 (HSV-2); (4) Mycobacterium tuberculosis (TB); and (5) Dengue virus type 3 (dengue fever).

[0225]

2122 Two diagnostically relevant regions from the HIV-1 genome, p17 and polyprotease, were selected from the Los Alamos National Laboratory HIV-1 reference sequence.

2124 Diagnostic sequences from the P. falciparum ChR 7 gene were obtained from the UCSC P. falciparum genome browser.

2126 Herpes simplex virus 2 sequences were synthesized from sequences obtained from the European Molecular Biology Library.

2128 Similarly, diagnostic sequences for the rpoB gene in Mycobacterium tuberculosis were synthesized from sequences obtained from the European Molecular Biology Library.

2130 PCR diagnostic sequences for dengue virus type 3 were obtained from the National Institute of Health's Health Gene Sequence Database.

[0226]

2135 Oligonucleotides are synthesized from INTEGRATED DNA TECHNOLOGIES (IDT).

2136 Probe and primer pairs for each analyte were selected using the OLIGO analyzer from IDT.

2137 Sensing probes containing a fluorophore at the 5' end and a quencher at the 3' end were synthesized for all

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analytes. Lyophilize all oligonucleotides and reconstitute in TE buffer before use. Tables 9-14 show the sequence information, target sequences, primers, and probes for each of the six analytes.

[0227]

2143 Table 9.

2144 Sequence information of HIV-1 polyprotease.

[0229]

2148 Table 10.

2149 Sequence information for HIV p17.

[0232]

2153 Table 11.

2154 Sequence information of Plasmodium ChR 7.

[0234]

2158 Table 12.

2159 Sequence information for herpes simplex virus 2.

[0236]

2163 Table 13.

2164 Sequence information of Mycobacterium tuberculosis rpoB.

[0239]

2168 Table 14.

2169 Sequence information of dengue virus type 3.

[0241]

2173 3.

2174 polymerase chain reaction

[0242]

2178 All PCR reactions were performed on a ROCHE 480LIGHTCYCLER instrument.

2179 Perform 45 cycles with a 60-second hot start at 95° C.

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2180 The cycle conditions for denaturation, annealing and extension were 45 seconds, 50 seconds and 60 seconds at 95° C, 65° C and 70° C respectively.

2182 Each experiment was run in five replicates with a reaction volume of 15 μL.

2183 After each annealing step, fluorescence measurements were obtained in the following channels: 483nm to 533nm (FAM), 523nm to 568nm (Cy3), 558nm to 610nm (ROX), and 615nm to 670nm (Cy5).

2185 Fluorescence measurements can also be obtained after a hot start and after 45 thermal cycles.

2186 The change in fluorescence intensity between these two measurements (after hot start, and at the end of 45 cycles) determines the endpoint fluorescence signal.

[0243]

2191 Example 2: Simultaneous detection of polynucleotide markers from pathogens using more than one color coding approach per analyte

[0244]

2196 This example describes the simultaneous detection of up to four polynucleotides selected from HIV (2 polynucleotides), HSV-2, Mycobacteria, Plasmodium, and Dengue virus.

2198 Fourteen PCR reactions were assembled using the reagents described in Table 15.

[0245]

2202 Table 15.

2203 Reagents for 14 PCR reactions.

[0251]

2207 Referring to Table 15, reactions 1, 3, 5, 7, and 9 (containing analytes for HIV polyprotease, Plasmodium, HSV, mycobacteria, and dengue, respectively) were calculated for each polynucleotide in the absence of other analytes. Analytes alone provide a positive control for baseline fluorescence intensity.

2210 As described more fully below, the change in fluorescence intensity in each color is used to provide the expected cumulative intensity level for each color in the color spectrum (Figure 3).

2212 Reactions 2, 4, 6, 8, and 10 are negative controls, respectively, used to determine the extent of nonspecific amplification.

2214 In these reactions, the primers provided are specific for the analyte provided.

[0252]

2218 Experiments 11, 12, 13 and 14 are multiplex detection experiments.

2219 The encoding is performed as shown in Table 16.

2220 Within each color, the fluorescence intensity measured for each assay is depicted as a black circle in the chromatogram of Figure 3.

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[0253]

2225 Table 16.

2226 Coding scheme for pathogen detection.

[0254]

2230 FAM Cy3 ROX Cy5 HIV polyprotease 1 0 0 0 HIV p17 1 1 0 0 Plasmodium 1 0 1 0 HSV 1 0 0 1 Mycobacterium 1 1 0 1 Dengue virus 1 1 1 1

[0255]

2235 To construct the chromatogram, in each color, the signals of the positive controls (reactions 1, 3, 5, 7 and 9) were used to assemble every possible combination of sequences present.

2237 The accumulated signal is plotted in a graph called a "chromatogram" (Figure 3).

2238 The accumulated signal corresponding to all possible combinations of the same rank in the same color is organized into its own "band".

2240 Doing this for all four colors produces level and band structures against which the experimental results for each combination can be evaluated (Reactions 11, 12, 13 and 14).

2242 Figure 3 shows three replicates of the chromatogram constructed using the positive control sample with the experimental results 4112, 3121 and 3102 superimposed on the chromatogram.

2244 Experimental results are indicated by black dots.

[0256]

2248 The intensity value of each color in reactions 11-14 was measured in arbitrary fluorescence units (AFU), plotted in a chromatogram, and then assigned to bands based on two criteria.

2250 First, if the experimental result is within a band, then the result is assigned to the multiplexed band (e.g., a Cy3 value of 1 in the leftmost chromatogram of Figure 3).

2252 Second, if the experimental results are between two bands, a two-step analysis is used.

2253 If the experimental result is between two reasonable results, then the experimental result is assigned to the closest band (e.g., the value 2 for Cy5 in the rightmost chromatogram of Figure 3).

2255 If the experimental result is between a reasonable result and an unreasonable result, the result is assigned to the reasonable result of the band (eg, FAM value 3 in the middle chromatogram of Figure 3). Figure 8 schematically shows a reasonable result for 4112 (top panel), which indicates the presence of HIV polyprotease, Plasmodium, herpes simplex, and mycobacteria; and an unreasonable result for 4110 (bottom panel), It cannot be decoded and indicates a failure in the measurement.

[0257]

2263 Using these criteria, the output of Experiment 12 is assigned 4112, which is the correct answer.

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2264 The output of Experiment 14 could be specified as 2121 or 3121, but 2121 is unreasonable, so the result must be 3121, which is the correct answer. The output of Experiment 13 could be assigned as 3102 or 3101, both of which are reasonable, but in the Cy5 channel the result is definitely closer to 2 than 1, so the correct answer, 3102, is assigned as the result. These results provide experimental validation of the method presented in this paper.

[0258]

2272 The results showed a tendency for the combined signal to be slightly lower than what would be expected from the simple sum of the signals from the positive controls.

2274 This may be because the combination of signals is not perfectly linear. This may be the result of additional effects of the chromatographic construct that are currently unexplained. These effects may be related to the absolute concentration of the quencher. The higher the absolute concentration of the quencher, the greater the percentage of the reaction volume it quenches, such that the same percentage of released fluorophore does not contribute as much to the cumulative signal as expected. One solution to this problem is to use a low concentration of probe such that the percent loss in signal is negligible, regardless of how much quencher is released.

[0259]

2284 Figure 3 shows that each rank contains multiple combinations with relatively broad bands.

2285 The width of the band is ultimately the difference between the highest and lowest accumulated signal of the positive control sample. If all positive controls produced exactly the same fluorescent signal in a particular color, the width of each band would be zero. Because the substituted signals are different, the resulting bands are relatively broad. However, there is a simple means to tighten the straps. As done in the experiment above, instead of loading all probes at the same concentration, the concentration of each probe can be adjusted so that each resulting positive control signal is the same as the others in the same color. This method significantly tightens the bands, making it easier to determine the multiplexing of the signal for samples containing the analyte.

[0260]

2296 Example 3: Simultaneous detection of polynucleotide markers from pathogens using one color and different intensity encoding methods per analyte

[0261]

2301 This example describes the simultaneous detection of up to three polynucleotides selected from dengue virus, HIV p17 and HIV polyprotease.

2303 The coding scheme is as depicted in Table 11.

2304 As shown in Table 11, each analyte is coded as a single value of fluorescence intensity in a single color. In this case, the encoding scheme is non-degenerate by design, as described elsewhere in this disclosure. This is

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accomplished by assigning an intensity value to each analyte equal to the cumulative intensity value of the previous analyte plus one. Of course, as described elsewhere in this specification, the initial assigned value may be greater than 1, for example, to better distinguish noise. Similarly, the value assigned to each analyte can be greater than the cumulative intensity value of the previous analyte plus one, for example, to increase the spacing between intensities and make intensity assignment simpler.

[0262]

2314 Table 17.

2315 Coding scheme for detection of dengue virus, HIV polyprotease, and HIV p17.

[0263]

2319 FAM Dengue virus 1 HIV p17 2 HIV polyprotease 4

[0264]

2323 The same reagents (eg, templates, primers, and probes) used in Example 2 were used here.

2324 The fluorescence signal produced by each probe in the positive control endpoint PCR reaction was measured and used to calculate the concentration of probes that would produce 1x, 2x, and 4x signal intensities.

2326 The experiment was then set up to detect seven non-empty combinations of analyte occurrences. Tables 18-24 provide the analyte PCR reaction components for each of these mixtures.

[0265]

2331 Table 18.

2332 Binary FAM experiment 1 cocktail (Cocktail): HIV polyprotease, HIV p17, and dengue virus.

[0267]

2336 Table 19.

2337 Binary FAM Experiment 2 Mix: Dengue Virus and HIV p17.

[0269]

2341 Table 20.

2342 Binary FAM Experiment 3 Mix: Dengue Virus and HIV Polyprotease.

[0271]

2346 Table 21.

2347 Binary FAM Experiment 4 Mix: HIV Polyprotease and HIV p17.

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[0274]

2351 Table 22.

2352 Binary FAM Experiment 5 Mix: Dengue Virus.

[0276]

2356 Table 23.

2357 Binary FAM Experiment 6 Mix: HIV p17.

[0279]

2361 Table 24.

2362 Binary FAM Experiment 7 Mix: HIV Polyprotease.

[0281]

2366 The total fluorescence signal for each experiment was plotted on a chromatogram constructed as described above (Figure 7).

2368 The width of the band surrounding each positive control measurement is 1x the uncertainty in the propagation of the measurement.

2370 This uncertainty is equal to the standard deviation of the fluorescence signal over the last five PCR cycles of saturation.

[0282]

2375 Figure 7 shows the results of this experiment.

2376 The three results on the far right show the measured intensities of Dengue virus alone (DEN), HIV p17 alone (P17), and HIV polyprotease (TPP).

2378 The assigned intensities for each of these analytes are shown in Table 11, and Figure 7 confirms that the three analytes, when present alone, produced the expected intensity results (see DEN, P17, and TPP).

2380 At the end of the assay, substantially all of the probe has been hydrolyzed, releasing substantially all of the fluorophores emitting signal.

2382 A fixed number of fluorophores produces a fixed and predictable amount of signal.

2383 Therefore, the presence of target should generally produce a fixed amount of signal, determined by the amount of probe added to the reaction volume.

[0283]

2388 As expected, the intensity value for a combination of analytes is equal to the sum of the assigned intensities.

2389 Because this coding scheme is designed to be nondegenerate, each result can be unambiguously decoded into

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the presence or absence of a specific analyte.

2391 For example, starting on the left side of Figure 7, the result for 7 indicates that all three analytes are present.

2392 The results for 3 indicate that dengue virus and HIV p17 analytes are present, but HIV polyprotease is not present.

2394 The results of 5 indicate that dengue virus and HIV polyprotease are present, but HIV p17 is not.

2395 Finally, the results of 6 indicate that HIV p17 and HIV polyprotease are present, but not dengue virus. This encoding scheme can be expanded to encode additional analytes as described elsewhere in this disclosure.

[0284]

2400 sequence list

[0285]

2404 <110> California Institute of Technology

[0286]

2408 <120> Encoding and decoding of signals in multiplex biochemical assays

[0287]

2412 <130> 38075-716.601

[0288]

2416 <140> PCT/US2013/024509

[0289]

2420 <141> 2013-02-01

[0290]

2424 <150> 61/703,093

[0291]

2428 <151> 2012-09-19

[0292]

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2432 <150> 61/594,480

[0293]

2436 <151> 2012-02-03

[0294]

2440 <160> 32

[0295]

2444 <170> PatentIn version 3.5

[0296]

2448 <210> 1

[0297]

2452 <211> 198

[0298]

2456 <212> DNA

[0299]

2460 <213> Human Immunodeficiency Virus 1

[0300]

2464 <400> 1

[0301]

2468 ggaagctcta ttagatacag gagcagatga tacagtatta gaagaaatga gtttgccagg 60

[0302]

2472 aagatggaaa ccaaaaatga tagggggaat tggaggtttt atcaaagtaa gacagtatga 120

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[0303]

2476 tcagatactc atagaaatct gtggacataa agctataggt acagtattag taggacctac 180

[0304]

2480 acctgtcaac ataattgg 198

[0305]

2484 <210> 2

[0306]

2488 <211> 26

[0307]

2492 <212> DNA

[0308]

2496 <213> Artificial sequence

[0309]

2500 <220>

[0310]

2504 <223> Description of artificial sequences: synthetic primers

[0311]

2508 <400> 2

[0312]

2512 ggaagctcta ttagatacag gagcag 26

[0313]

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2516 <210> 3

[0314]

2520 <211> 26

[0315]

2524 <212> DNA

[0316]

2528 <213> Artificial sequence

[0317]

2532 <220>

[0318]

2536 <223> Description of artificial sequences: synthetic primers

[0319]

2540 <400> 3

[0320]

2544 ccaattatgt tgacaggtgt aggtcc 26

[0321]

2548 <210> 4

[0322]

2552 <211> 26

[0323]

2556 <212> DNA

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[0324]

2560 <213> Artificial sequence

[0325]

2564 <220>

[0326]

2568 <223> Description of artificial sequences: synthetic probes

[0327]

2572 <220>

[0328]

2576 <223> 5'6-FAM

[0329]

2580 <220>

[0330]

2584 <223> 3'BHQ_1

[0331]

2588 <400> 4

[0332]

2592 tgagtttgcc aggaagatgg aaacca 26

[0333]

2596 <210> 5

[0334]

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2600 <211> 199

[0335]

2604 <212> DNA

[0336]

2608 <213> Human Immunodeficiency Virus 1

[0337]

2612 <400> 5

[0338]

2616 cagctacaac catcccttca gacaggatca gaagaactta gatcattata taatacagta 60

[0339]

2620 gcaaccctct attgtgtgca tcaaaggata gagataaaag acaccaagga agctttagac 120

[0340]

2624 aagatagagg aagagcaaaa caaaagtaag aaaaaagcac agcaagcagc agctgacaca 180

[0341]

2628 ggacacagca atcaggtca 199

[0342]

2632 <210> 6

[0343]

2636 <211> 24

[0344]

2640 <212> DNA

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[0345]

2644 <213> Artificial sequence

[0346]

2648 <220>

[0347]

2652 <223> Description of artificial sequences: synthetic primers

[0348]

2656 <400> 6

[0349]

2660 cagctacaac catcccttca gaca 24

[0350]

2664 <210> 7

[0351]

2668 <211> 24

[0352]

2672 <212> DNA

[0353]

2676 <213> Artificial sequence

[0354]

2680 <220>

[0355]

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2684 <223> Description of artificial sequences: synthetic primers

[0356]

2688 <400> 7

[0357]

2692 tgacctgatt gctgtgtcct gtgt 24

[0358]

2696 <210> 8

[0359]

2700 <211> 28

[0360]

2704 <212> DNA

[0361]

2708 <213> Artificial sequence

[0362]

2712 <220>

[0363]

2716 <223> Description of artificial sequences: synthetic probes

[0364]

2720 <220>

[0365]

2724 <223> 5'6-FAM

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[0366]
2728 <220>

[0367]
2732 <223> 3'BHQ_1

[0368]
2736 <400> 8

[0369]
2740 agcaaccctc tattgtgtgc atcaaagg 28

[0370]
2744 <210> 9

[0371]
2748 <211> 23

[0372]
2752 <212> DNA

[0373]
2756 <213> Artificial sequence

[0374]
2760 <220>

[0375]
2764 <223> Description of artificial sequences: synthetic probes

[0376]

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2768 <220>

[0377]

2772 <223> 5'Cy3

[0378]

2776 <220>

[0379]

2780 <223> 3'BHQ_2

[0380]

2784 <400> 9

[0381]

2788 aaagcacagc aagcagcagc tga 23

[0382]

2792 <210> 10

[0383]

2796 <211> 199

[0384]

2800 <212> DNA

[0385]

2804 <213> Plasmodium falciparum

[0386]

2808 <400> 10

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[0387]

2812 gcctaacatg gctatgacgg gtaacgggga attagagttc gattccggag agggagcctg 60

[0388]

2816 agaaatagct accacatcta aggaaggcag caggcgcgta aattacccaa ttctaaagaa 120

[0389]

2820 gagaggtagt gacaagaaat aacaatgcaa ggccaattta aaaccttccc agagtaacaa 180

[0390]

2824 ttggagggca agtctggtg 199

[0391]

2828 <210> 11

[0392]

2832 <211> 24

[0393]

2836 <212> DNA

[0394]

2840 <213> Artificial sequence

[0395]

2844 <220>

[0396]

2848 <223> Description of artificial sequences: synthetic primers

[0397]

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2852 <400> 11

[0398]

2856 gcctaacatg gctatgacgg gtaa 24

[0399]

2860 <210> 12

[0400]

2864 <211> 24

[0401]

2868 <212> DNA

[0402]

2872 <213> Artificial sequence

[0403]

2876 <220>

[0404]

2880 <223> Description of artificial sequences: synthetic primers

[0405]

2884 <400> 12

[0406]

2888 caccagactt gccctccaat tgtt 24

[0407]

2892 <210> 13

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[0408]
2896 <211> 26

[0409]
2900 <212> DNA

[0410]
2904 <213> Artificial sequence

[0411]
2908 <220>

[0412]
2912 <223> Description of artificial sequences: synthetic probes

[0413]
2916 <220>

[0414]
2920 <223> 5'6-FAM

[0415]
2924 <220>

[0416]
2928 <223> 3'BHQ_1

[0417]
2932 <400> 13

[0418]

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2936 attccggaga gggagcctga gaaata 26

[0419]

2940 <210> 14

[0420]

2944 <211> 26

[0421]

2948 <212> DNA

[0422]

2952 <213> Artificial sequence

[0423]

2956 <220>

[0424]

2960 <223> Description of artificial sequences: synthetic probes

[0425]

2964 <220>

[0426]

2968 <223> 5'6-ROXN

[0427]

2972 <220>

[0428]

2976 <223> 3'BHQ_2

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[0429]

2980 <400> 14

[0430]

2984 aaggaaggca gcaggcgcgt aaatta 26

[0431]

2988 <210> 15

[0432]

2992 <211> 192

[0433]

2996 <212> DNA

[0434]

3000 <213> Herpes simplex virus 2

[0435]

3004 <400> 15

[0436]

3008 tcagcccatc ctccttcggc agtatggagg gtgtcgcggc ggcgagccgc cgtccccaaa 60

[0437]

3012 gacgtgcggg tcgtacacgt acacgtacca gggcggcggg cctccgaccc ggtacgctct 120

[0438]

3016 cgtaaatgct tccctgctgg tgccgatctg ggaccgcgcc gcggagacat tcgagtacca 180

[0439]

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3020 gatcgaactcgg 192

[0440]

3024 <210> 16

[0441]

3028 <211> 25

[0442]

3032 <212> DNA

[0443]

3036 <213> Artificial sequence

[0444]

3040 <220>

[0445]

3044 <223> Description of artificial sequences: synthetic primers

[0446]

3048 <400> 16

[0447]

3052 tcagcccatc ctccttcggc agtat 25

[0448]

3056 <210> 17

[0449]

3060 <211> 26

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[0450]

3064 <212> DNA

[0451]

3068 <213> Artificial sequence

[0452]

3072 <220>

[0453]

3076 <223> Description of artificial sequences: synthetic primers

[0454]

3080 <400> 17

[0455]

3084 ccgagttcga tctggtactc gaatgt 26

[0456]

3088 <210> 18

[0457]

3092 <211> 26

[0458]

3096 <212> DNA

[0459]

3100 <213> Artificial sequence

[0460]

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3104 <220>

[0461]

3108 <223> Description of artificial sequences: synthetic probes

[0462]

3112 <220>

[0463]

3116 <223> 5'6-FAM

[0464]

3120 <220>

[0465]

3124 <223> 3'BHQ_1

[0466]

3128 <400> 18

[0467]

3132 aaagacgtgc gggtcgtaca cgtaca 26

[0468]

3136 <210> 19

[0469]

3140 <211> 25

[0470]

3144 <212> DNA

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[0471]

3148 <213> Artificial sequence

[0472]

3152 <220>

[0473]

3156 <223> Description of artificial sequences: synthetic probes

[0474]

3160 <220>

[0475]

3164 <223> 5' Cy5

[0476]

3168 <220>

[0477]

3172 <223> 3'3IAbRQSp

[0478]

3176 <400> 19

[0479]

3180 taaatgcttc cctgctggtg ccgat 25

[0480]

3184 <210> 20

[0481]

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3188 <211> 200

[0482]

3192 <212> DNA

[0483]

3196 <213> Mycobacterium tuberculosis

[0484]

3200 <400> 20

[0485]

3204 gagtgcaaag acaaggacat gacgtacgcg gccccgctgt tcgtcacggc cgagttcatc 60

[0486]

3208 aacaacaaca ccggcgagat caagagccag acggtgttca tgggtgactt cccgatgatg 120

[0487]

3212 accgagaagg gcaccttcat catcaacggc accgagcgcg tcgtggtcag ccagctggtc 180

[0488]

3216 cgctcgcccg gtgtgtactt 200

[0489]

3220 <210> 21

[0490]

3224 <211> 24

[0491]

3228 <212> DNA

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[0492]
3232 <213> Artificial sequence

[0493]
3236 <220>

[0494]
3240 <223> Description of artificial sequences: synthetic primers

[0495]
3244 <400> 21

[0496]
3248 gagtgcaaag acaaggacat gacg 24

[0497]
3252 <210> 22

[0498]
3256 <211> 19

[0499]
3260 <212> DNA

[0500]
3264 <213> Artificial sequence

[0501]
3268 <220>

[0502]

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3272 <223> Description of artificial sequences: synthetic primers

[0503]

3276 <400> 22

[0504]

3280 aagtacacac cgggcgagc 19

[0505]

3284 <210> 23

[0506]

3288 <211> 25

[0507]

3292 <212> DNA

[0508]

3296 <213> Artificial sequence

[0509]

3300 <220>

[0510]

3304 <223> Description of artificial sequences: synthetic probes

[0511]

3308 <220>

[0512]

3312 <223> 5'6-FAM

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[0513]
3316 <220>

[0514]
3320 <223> 3'BHQ_1

[0515]
3324 <400> 23

[0516]
3328 cgctgttcgt cacggccgag ttcat 25

[0517]
3332 <210> 24

[0518]
3336 <211> 26

[0519]
3340 <212> DNA

[0520]
3344 <213> Artificial sequence

[0521]
3348 <220>

[0522]
3352 <223> Description of artificial sequences: synthetic probes

[0523]

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3356 <220>

[0524]

3360 <223> 5'Cy3

[0525]

3364 <220>

[0526]

3368 <223> 3'BHQ_2

[0527]

3372 <400> 24

[0528]

3376 agatcaagag ccagacggtg ttcatg 26

[0529]

3380 <210> 25

[0530]

3384 <211> 25

[0531]

3388 <212> DNA

[0532]

3392 <213> Artificial sequence

[0533]

3396 <220>

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[0534]

3400 <223> Description of artificial sequences: synthetic probes

[0535]

3404 <220>

[0536]

3408 <223> 5' Cy5

[0537]

3412 <220>

[0538]

3416 <223> 3' IAbRQSp

[0539]

3420 <400> 25

[0540]

3424 aagggcacct tcatcatcaa cggca 25

[0541]

3428 <210> 26

[0542]

3432 <211> 200

[0543]

3436 <212> DNA

[0544]

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3440 <213> Dengue virus 3

[0545]

3444 <400> 26

[0546]

3448 atgccaactg tgattgagca cttagaaaga ctacaaagga aacatggagg aatgcttgtg 60

[0547]

3452 agaaatccac tctcacgaaa ctccacgcac gaaatgtatt ggatatccaa tggtacaggc 120

[0548]

3456 aacatcgtct cattcacaat gacacacagg agacccacca tagagaaaga tgtggattta 180

[0549]

3460 ggagcaggaa cccgacatgt 200

[0550]

3464 <210> 27

[0551]

3468 <211> 22

[0552]

3472 <212> DNA

[0553]

3476 <213> Artificial sequence

[0554]

3480 <220>

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[0555]

3484 <223> Description of artificial sequences: synthetic primers

[0556]

3488 <400> 27

[0557]

3492 atgccaactg tgattgagca ct 22

[0558]

3496 <210> 28

[0559]

3500 <211> 24

[0560]

3504 <212> DNA

[0561]

3508 <213> Artificial sequence

[0562]

3512 <220>

[0563]

3516 <223> Description of artificial sequences: synthetic primers

[0564]

3520 <400> 28

[0565]

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3524 acatgtcggg ttcctgctcc taaa 24

[0566]

3528 <210> 29

[0567]

3532 <211> 29

[0568]

3536 <212> DNA

[0569]

3540 <213> Artificial sequence

[0570]

3544 <220>

[0571]

3548 <223> Description of artificial sequences: synthetic probes

[0572]

3552 <220>

[0573]

3556 <223> 5'6-FAM

[0574]

3560 <220>

[0575]

3564 <223> 3'BHQ_1

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[0576]

3568 <400> 29

[0577]

3572 acaaaggaaa catggaggaa tgcttgtga 29

[0578]

3576 <210> 30

[0579]

3580 <211> 26

[0580]

3584 <212> DNA

[0581]

3588 <213> Artificial sequence

[0582]

3592 <220>

[0583]

3596 <223> Description of artificial sequences: synthetic probes

[0584]

3600 <220>

[0585]

3604 <223> 5' Cy3

[0586]

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3608 <220>

[0587]

3612 <223> 3'BHQ_2

[0588]

3616 <400> 30

[0589]

3620 actctcacga aactccacgc acgaaa 26

[0590]

3624 <210> 31

[0591]

3628 <211> 26

[0592]

3632 <212> DNA

[0593]

3636 <213> Artificial sequence

[0594]

3640 <220>

[0595]

3644 <223> Description of artificial sequences: synthetic probes

[0596]

3648 <220>

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[0597]

3652 <223> 5'6-ROXN

[0598]

3656 <220>

[0599]

3660 <223> 3'BHQ_2

[0600]

3664 <400> 31

[0601]

3668 acaatgacac acaggagacc caccat 26

[0602]

3672 <210> 32

[0603]

3676 <211> 28

[0604]

3680 <212> DNA

[0605]

3684 <213> Artificial sequence

[0606]

3688 <220>

[0607]

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3692 <223> Description of artificial sequences: synthetic probes

[0608]

3696 <220>

[0609]

3700 <223> 5' Cy5

[0610]

3704 <220>

[0611]

3708 <223> 3'IAbRQSp

[0612]

3712 <400> 32

[0613]

3716 ggatatccaa tggtacaggc aacatcgt 28

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Espacenet

CN107326085A Signal encoding and decoding in multiplexed biochemical assays

Applicants: CALIFORNIA INST OF TECHN

Inventors: KARTALOV EMIL,RAJAGOPAL ADITYA,SCHERER AXEL

Classifications:

IPC ***G06F19/12; G06F19/20; C12Q1/6825; G16B45/00;***

CPC **C12Q1/68 (CN,KR,US); C12Q1/6825 (EP,CN,KR,US); C12Q1/6851 (EP,US); G01N21/6486 (US); G16B25/00 (CN,US); G16B25/20 (EP,CN,KR,US); G16B30/00 (KR); G16B40/10 (US); G16B45/00 (KR); G16B5/00 (CN,KR,US);** G06F17/10 (CN,US); G06F17/11 (CN,US); G16B40/00 (CN,US); G16B40/10 (EP,CN,KR); Y02A50/30 (US);

C-sets **C12Q1/68, C12Q2537/101, C12Q2537/143, C12Q2537/165, C12Q2563/107 (CN); C12Q1/6825, C12Q2537/101, C12Q2537/143, C12Q2537/165, C12Q2563/107 (CN); C12Q1/6851, C12Q2537/101, C12Q2537/143, C12Q2537/165, C12Q2563/107 (EP,US);**

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Application: CN201710674587A·2013-02-01

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Signal encoding and decoding in multiplexed biochemical assays

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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G06F 19/20(2011.01)

权利要求书1页 说明书49页
序列表9页 附图8页

(54)发明名称

多路生化测定中信号的编码和解码

(57)摘要

本公开提供了用于样品中的多个分析物的多路检测的方法、系统、组合物、和试剂盒。在一些实例中，本公开提供了方法、系统、组合物、和试剂盒，其中，可在单个样品体积中通过获得信号的至少一个可计量的分量的累积测量或多个累积测量来检测多个分析物。在一些情况下，信号的额外分量、或额外的信号(或其分量)也被定量。每个信号或信号的每个分量可被用于构建编码方案，然后所述编码方案可用于确定任何分析物的存在或不存在。

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1.一种方法，所述方法能够不进行固定化、分离、质谱法、或解链曲线分析而非简并地检测单一样品体积中的、以存在或不存在的任意组合形式的至少7个分析物中的每个分析物的存在或不存在，其中所述方法包括：

(i)将待检测的每个分析物编码为信号的第一分量中的至少一个第一值和所述信号的第二分量中的至少一个第二值；

(ii)枚举可以从所述编码方案获得的至少一个结果；

(iii)鉴定是简并的至少一个结果；和

(iv)从所述编码方案消除至少一个潜在的分析物代码，其中所述消除至少一个潜在的分析物代码减少或消除所述编码方案的结果的简并性。

2.根据权利要求1所述的方法，其中，所述方法能够明确地检测以存在或不存在的任意组合形式的M个分析物的存在或不存在，其中$M=\log_2(F+1)$，且F是当所有所述分析物存在时的所述信号的所述一个分量的最大累积值。

3.根据权利要求1所述的方法，其中，所述方法能够明确地检测以存在或不存在的任意组合形式的M个分析物的存在或不存在，其中$M=C*\log_2(F+1)$，其中C是用于编码所述分析物的所述第二值的数目，且F是当所有所述分析物存在时，关于任意第二值的所述信号的所述第一分量的最大累积值。

4.根据权利要求1所述的方法，其中，所述方法能够明确地检测以存在或不存在的任意组合形式的M个分析物的存在或不存在，其中$M=(P*T)+1$，其中P是编码方案中每层代码的数目，且T是层的数目。

5.根据权利要求4所述的方法，其中，所述层的数目$T=\log4(F+1)$，且F是当所有所述分析物存在时，关于任意第二值的所述信号的所述第一分量的最大累积值。

6.根据权利要求1所述的方法，其中，所述第一值是强度或强度的范围。

7.根据权利要求4所述的方法，其中，所述编码方案包括至少三个强度或强度的范围。

8.根据权利要求1所述的方法，其中，所述第二值是波长或波长的范围。

9.根据权利要求8所述的方法，其中，所述编码方案包括至少五个波长或波长的范围。

10.根据权利要求1所述的方法，其中，所述信号是电磁信号。

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多路生化测定中信号的编码和解码

[0001] 本申请是申请日为2013年2月1日，申请号为201380018760.9，发明名称为“多路生化测定中信号的编码和解码”的申请的分案申请。

[0002] 交叉引用

[0003] 本申请要求2012年2月3日提交的美国临时申请61/594,480，及2012年9月19日提交的美国临时申请61/703,093的利益，其各自通过引用其全文并入本文。

[0004] 序列表

[0005] 本即时申请包含经由EFS-网页以ASCII格式提交的序列表，且通过引用将其全文并入本文。所述ASCII副本，建立于2013年2月14日，被命名为38075-716.601_SL.txt，且大小为9,240字节。

技术领域

[0006] 本发明涉及多路生化测定中信号的编码和解码。

背景技术

[0007] 相比传统的单路反应，多路反应提供了显著的优点，包括在相同样品上执行并行的多个反应，使用相同的室执行更多个反应，以及以快速且有效的方式从样品中提取丰富信息的能力。然而，为了实现这些优点，多路测定通常需要复杂的报告机制，即光谱上可分辨的荧光或化学发光(例如，PCR、ELISA)、空间上可分辨的信号(例如，微阵列、凝胶电泳)、时间上可分辨的信号(例如，毛细管电泳)或其组合(例如，Sanger测序)。

发明内容

[0008] 对可以在单一的溶液中执行的多路反应是有需求的。

[0009] 本公开提供了用于分析物的多路检测的方法、组合物、系统和试剂盒。在一些情况下，本公开提供测定，所述测定能够不执行固定化(immobilization)、分离、质谱法、或解链(melting)曲线分析而明确地检测单一样品体积中的、以存在或不存在的任意组合形式的至少7种分析物中的每种分析物的存在或不存在。在一些实例中，每种分析物被编码为信号的一个(或至少一个)分量的值。

[0010] 在一些实例中，本公开提供的测定能够明确地检测以存在或不存在的任意组合形式的、M种分析物的存在或不存在，其中$M=\log_2(F+1)$，且F是当所有分析物存在时的信号的一个分量(例如，强度)的最大累积值。

[0011] 在一些情况下，每种分析物被编码为信号的第一分量中的至少一个第一值(例如，至少一个强度或强度范围)和信号的第二分量中的至少一个第二值(例如，至少一个波长或波长范围)。在一些实例中，每种分析物被编码为在信号的第二分量中的多个第二值中的每个第二值的信号的第一分量中的第一值(例如，在多个波长或多个波长范围中的每个波长或每个波长范围的信号强度或信号强度范围)。

[0012] 在一些实例中，本文提供的测定能够明确地检测以存在或不存在的任意组合形式

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的、M种分析物的存在或不存在，其中M＝C*$\log_2$(F+1)，其中C是用于编码分析物的第二值的数目，且F是在所有分析物存在时的、关于任意第二值的信号的第一分量的最大累积值。

[0013] 在一些情况下，本公开提供的测定能够明确地检测以存在或不存在的任意组合形式的、M种分析物的存在或不存在，其中M＝(P*T)+1，其中P是编码方案中每层代码的数目，且T是层的数目。在一些情况下，层的数目T＝$\log_4$(F+1)，且F是在所有分析物存在时的、关于任意第二值的信号的第一分量的最大累积值。

[0014] 在一些实例中，第一值是强度或强度范围。在一些情况下，编码方案包括至少三个强度或强度范围。

[0015] 在一些情况下，第二值是波长或波长范围。在一些实例中，编码方案包括至少五个波长或波长范围。

[0016] 在一些实施例中，信号是电磁信号。在一些情况下，电磁信号是荧光发射信号。在一些实施例中，测量了在至少四个波长或波长范围处的荧光发射信号的强度。

[0017] 在一些情况下，本文提供的测定利用在使用之前被冻干的试剂执行。

[0018] 在一些情况下，利用包括杂交探针的试剂执行检测步骤。在一些实例中，杂交探针的数目大于分析物的数目。在一些情况下，杂交探针组包括专门用于不同分析物的一个或更多个杂交探针，且包括相同的荧光团或荧光团的组合。在一些实例中，样品与至少18个所述杂交探针接触。在一些实例中，利用包括至少一对引物的试剂执行检测步骤。在一些情况下，至少一对引物能够扩增与至少三个所述杂交探针互补的区域。

[0019] 在一些实例中，在聚合酶链式反应期间产生被测量的信号。在一些情况下，聚合酶链式反应选自以下所构成的组：终点(end-point)聚合酶链式反应、实时聚合酶链式反应、数字聚合酶链式反应、及其组合。

[0020] 在一些实例中，针对溶液执行至少一个累积测量。

[0021] 在一些实例中，至少一种分析物是通过至少一个额外的值(即，至少两个值一起)编码的，其中，至少一个额外的值选自以下所构成的组：来自信号的至少一个额外的分量的值，来自不同的信号的至少一个分量的值，及其组合。例如，至少一个额外的值可选自以下所构成的组：荧光发射强度、荧光发射波长、Förster共振能量转移(FRET)发射强度、FRET发射波长、电化学信号、化学发光波长、化学发光强度、荧光漂白率、化学发光漂白率、及其组合。

[0022] 在一些情况下，色谱被构建。通过绘制每个分析物的阳性对照(positive control)样品的第一值和第二值的所有可能的组合可构建色谱。

[0023] 在一些实例中，至少一个分析物包括多核苷酸。在一些情况下，多核苷酸来自选自以下所构成的组的来源：动物、植物、细菌、真菌、寄生物和病毒。在一些实例中，多核苷酸来自选自以下所构成的组的来源：人类免疫缺陷病毒、单纯性疱疹病毒、人类乳头瘤病毒、疟原虫、分枝杆菌、登革热病毒、肝炎病毒和流感病毒。在一些情况下，多核苷酸选自以下所构成的组：人类免疫缺陷病毒多蛋白酶(polyprotease)、人类免疫缺陷病毒p17、人类乳头瘤病毒E6、及人类乳头瘤病毒E7。

[0024] 在一些情况下，样品选自以下所构成的组：临床样品、食品样品、环境样品、药物样品、及来自消费产品的样品。

[0025] 在一些实例中，关于分析物存在或不存在的信息通过计算机网络传输。

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[0026] 在一些情况下，向医生提供关于分析物存在或不存在的信息。在一些实例中，临床决定是基于此类信息做出的。

[0027] 在一些实例中，本文提供的方法的至少一个步骤是使用计算机可读介质上的指令执行的。在一些情况下，指令位于远程服务器上。在一些实例中，指令位于热循环仪上。在一些情况下，指令位于与热循环仪通信的计算机上。

[0028] 在一些情况下，至少一个分析物是阳性对照分析物。

[0029] 在一些实例中，编码方案是非简并的。在一些情况下，编码方案被设计为非简并的。在一些实例中，通过枚举可以从编码方案获得的每个合理的结果、鉴定是简并的每个合理的结果、并消除来自编码方案的至少一个潜在的分析物代码以消除简并性，编码方案被做成非简并的。

[0030] 在一些情况下，本文提供的测定是终点测定。在一些实例中，本公开提供的测定在通过仪器的检测极限设定的循环的阈值数结束。

[0031] 在一些实例中，本文提供的测定是液相测定。

[0032] 在一些情况下，本文提供的测定是定量的。

[0033] 在一些情况下，本公开提供了检测多个分析物中的每个分析物存在或不存在的方法，所述方法包括：(a)将每个所述分析物编码为信号的第一值，由此产生编码方案，其中每个所述分析物在所述编码方案中由所述第一值表示，其中所述编码是以消除简并性的方式执行的；(b)提供包括或可能包括至少一个所述分析物的样品；(c)将所述样品与如所述编码方案中表示的在每个所述分析物存在时产生所述第一值的分析物特异性试剂接触；(d)累积地测量所述样品中所述信号的所述第一值，从而提供累积测量结果；及(e)基于所述累积测量结果和所述编码方案，确定每个所述分析物是存在还是不存在。

[0034] 在一些实例中，在先前段落描述的方法能够明确地检测以存在或不存在的任意组合的M种分析物的存在或不存在，其中$M=\log_2(F+1)$，且F是当所有分析物存在时的第一值的最大累积值。在一些情况下，第一值是强度或强度范围。在一些实例中，第一值具有在编码方案中的最小值，所述最小值选自以下所构成的组：至少1、至少2、至少4、至少8、至少16、至少32和至少64。在一些情况下，第一值通过等于所述先前第一值的累积最大值的加一的量在编码方案中增加。在其它情况下，第一值通过大于先前第一值的累积最大值加一的量在编码方案中增加。

[0035] 在一些情况下，本公开提供了检测多个分析物中的每个分析物存在或不存在的方法，所述方法包括：(a)以至少一个第一值和至少一个第二值编码每个所述分析物，其中所述第一值是来自信号的第一分量的值且所述第二值是来自所述信号的第二分量的值，从而产生编码方案，其中每个所述分析物在所述编码方案中通过所述至少一个第一值和所述至少一个第二值表示，其中所述编码以减少或消除简并性的方式执行；(b)提供包括或可能包括至少一种所述分析物的样品；(c)将所述样品与所述编码方案中表示的在每个所述分析物存在时产生所述至少一个第一值和至少一个所述第二值的分析物特异性试剂接触；(d)累积地测量所述样品中的所述第一值和所述第二值，从而提供累积测量结果；及(e)基于所述累积测量结果和所述编码方案，确定每个所述分析物是存在还是不存在，其中，当简并性被消除时，在每个所述试剂仅产生一个第二值时，所述方法能够明确地检测单一体积中的、以存在或不存在的任意组合形式的、至少6个分析物的每个分析物的存在或不存在。

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[0036] 在一些情况下，在先前段落描述的方法中的每个分析物被编码为在信号的第二分量中的多个第二值中的每个第二值的信号的第一分量中的第一值。在一些情况下，当简并性被消除时，该方法使用4个第二值能够明确地检测单一体积中的至少7个分析物中的每个分析物的存在或不存在。在一些实例中，当简并性被消除时，编码方案包括T个非简并层，其中$T=\log_4(F+1)$，且F是当所有分析物存在时，关于任意第二值的信号的第一分量的最大累积值。在一些情况下，当简并性被消除时，方法能够明确地检测以存在或不存在的任意组合形式的、M种分析物的存在或不存在，其中$M=(P*T)+1$，且P是每层代码的数目。在一些实例中，当简并性被消除时，方法能够明确地检测以存在或不存在的任意组合形式的、M种分析物的存在或不存在，其中$M=C*\log_2(F+1)$，C是编码方案中第二值的数目，且F是当所有分析物存在时，关于任意第二值的信号的第一分量的最大累积值。

[0037] 本公开还提供了非简并编码方案，所述非简并编码方案能够不执行固定化、分离、质谱法、或解链曲线分析而明确地编码单一样品体积中的、以存在或不存在的任意组合的至少7个分析物中的每个分析物的存在或不存在。

[0038] 在一些实例中，本公开的非简并编码方案是通过包括以下步骤的方法产生的：(a)产生用于每个潜在分析物的代码，其中每个潜在的分析物通过信号的至少一个分量的至少一个值编码；(b)枚举对存在或不存在的每个分析物的所有可能组合的每个合理的累积结果；(c)鉴定每个合理的简并的结果；及(d)消除至少一个代码，以消除简并性。在一些情况下，编码方案可通过增加额外的代码扩大，所述额外的编码比所述信号的至少一种分量中的所有先前的代码的总和大至少一个单位。在一些情况下，编码方案通过由结构保证非简并性的数学迭代的方法产生。

[0039] 在一些情况下，本公开提供了用于检测多个分析物中的每个分析物存在或不存在的系统，所述系统包括：(a)编码每个所述分析物为信号的第一值，由此产生编码方案，其中每个所述分析物在所述的编码方案中由所述第一值表示，其中所述编码是以消除简并性的方式执行的；(b)提供包括或可能包括至少一个所述分析物的样品；(c)将所述样品与如在所述编码方案中表示的、当每个所述分析物存在时产生所述第一值的分析物特异性试剂接触；(d)累积地在所述样品中测量所述信号的所述第一值，从而提供累积测量结果；及(e)基于所述累积测量结果和所述编码方案，确定每个所述分析物是存在还是不存在。

[0040] 在一些情况下，本公开提供了用于检测多个分析物中的每个分析物存在或不存在的系统，所述系统包括：(a)以至少一个第一值和至少一个第二值编码每个所述分析物，其中所述第一值是来自信号的第一分量的值且所述第二值是来自所述信号的第二分量的值，从而产生编码方案，其中每个所述分析物在所述编码方案中通过所述至少一个第一值和所述至少一个第二值表示，其中所述编码以减少或消除简并性的方式执行；(b)提供包括或可能包括至少一种所述分析物的样品；(c)将所述样品与在所述编码方案中表示的、当每个所述分析物存在时产生所述至少一个第一值和所述至少一个第二值的分析物特异性试剂接触；(d)累积地测量所述样品中的所述第一值和所述第二值，从而提供累积测量结果；及(e)基于所述累积测量结果和所述编码方案，确定每个所述分析物是存在还是不存在，其中，当简并性被消除时，在每个所述试剂仅产生一个第二值时，所述方法能够明确地检测在单一体积中的、以存在或不存在的任意组合形式的、至少6个分析物的每个分析物的存在或不存在。

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[0041] 在一些情况下，本公开提供了用于第三团体的检测多个分析物中的每个分析物存在或不存在的方法，所述方法包括：(a)从团体获得每个所述分析物的标识；(b)将每个所述分析物编码为信号的第一值，由此产生编码方案，其中每个所述分析物在所述的编码方案中由所述第一值表示，其中所述编码以消除简并性的方式执行；(c)向所述团体提供如在所述编码方案中表示的、当每个所述分析物存在时产生所述第一值的分析物特异性试剂，所述团体：(i)将包括或可能包括至少一个所述分析物的样品与所述试剂接触；及(ii)累积地测量所述样品中的所述第一值，从而提供累积测量结果；及(d)从所述团体获得所述累积测量结果；(e)基于所述累积测量结果和所述编码方案，确定每个所述分析物是存在还是不存在；及(f)将关于每个所述分析物存在或不存在的信息提供给所述团体。

[0042] 在一些情况下，本公开提供了用于第三团体的检测多个分析物中的每个分析物存在或不存在的方法，所述方法包括：(a)从团体获得每个所述分析物的标识；(b)将每个所述分析物编码为至少一个第一值和至少一个第二值，其中，所述第一值是来自信号的第一分量的值，且所述第二值是来自所述信号的第二分量的值，由此产生编码方案，其中每个所述分析物在所述的编码方案中由所述至少一个第一值和所述至少一个第二值表示，其中所述编码以减少或者消除简并性的方式执行；(c)向所述团体提供如所述编码方案中表示的在每个所述分析物存在时产生所述至少一个第一值和所述至少一个第二值的分析物特异性试剂，所述团体：(i)将包括或可能包括至少一个所述分析物的样品与所述试剂接触；及(ii)累积地测量所述样品中所述第一值及所述第二值，从而提供累积测量结果；及(d)从所述团体获得所述累积测量结果；(e)基于所述累积测量结果和所述编码方案，确定每个所述分析物是存在还是不存在；及(f)将关于每个所述分析物存在或不存在的信息提供给所述团体。

[0043] 在一些情况下，本公开提供了用于检测多个分析物中的每个分析物的存在或不存在的组合物，所述组合物包括分析物特异性试剂，每个试剂产生包括第一值的信号，其中所述试剂能够在不执行固定化、分离、质谱法、或解链曲线分析的情况下，明确地检测单一样品体积中的、以存在或不存在的任意组合形式的、至少7个分析物的每个分析物的存在或不存在。

[0044] 在一些情况下，本公开提供了用于检测多个分析物中的每个分析物的存在或不存在的组合物，所述组合物包括分析物特异性试剂，每个试剂产生包括至少一个第一值和至少一个第二值的信号，所述至少一个第一值是来自所述信号的第一分量的值且所述至少一个第二值是来自所述信号的第二分量的值，其中，当每个所述试剂仅产生一个第二值时，所述试剂能够检测在单一体积中的、以存在或不存在的任意组合形式的、至少6个分析物的每个分析物的存在或不存在。

[0045] 在一些情况下，本公开提供了用于检测多个分析物中的每个分析物的存在或不存在的试剂盒，所述试剂盒包括分析物特异性试剂、包装、及说明书，每个试剂产生包括第一值的信号，其中所述试剂盒能够在不执行固定化、分离、质谱法、或解链曲线分析的情况下，明确地检测单一样品体积中的、以存在或不存在的任意组合形式的、至少7个分析物的每个分析物的存在或不存在。

[0046] 在一些实例中，本公开提供了用于检测多个分析物中的每个分析物的存在或不存在的试剂盒，所述试剂盒包括分析物特异性试剂、包装、及说明书，每个试剂产生包括至少

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一个第一值和至少一个第二值的信号，所述至少一个第一值是来自所述信号的第一分量的值且所述至少一个第二值是来自所述信号的第二分量的值，其中，当每个所述试剂仅产生一个第二值时，所述试剂盒能够检测在单一体积中的、以存在或不存在的任意组合形式的、至少6个分析物中的每个分析物的存在或不存在。

[0047] 在一些情况下，本公开提供了用于检测多个分析物中的每个分析物存在或不存在的试剂盒，所述试剂盒包括盒体601、布置在盒体中用于放置瓶的卡接槽口、包括用于分析物的检测的探针的瓶602、包括用于扩增的引物的瓶603、及包括用于扩增的试剂的瓶604、其中，所述试剂盒能够在不执行固定化、分离、质谱法、或解链曲线分析的情况下，明确地检测单一样品体积中的、以存在或不存在的任意组合形式的至少7个分析物中的每个分析物的存在或不存在。

[0048] 在一些实例中，本公开提供了用于检测多个分析物中的每个分析物存在或不存在的试剂盒，包括：盒体601、布置在盒体中用于放置瓶的卡接槽口、包括用于分析物的检测的探针的瓶602、包括用于扩增的引物的瓶603、及包括用于扩增的试剂的瓶604，每个探针产生包括至少一个第一值和至少一个第二值的信号，所述至少一个第一值是来自所述信号的第一分量的值且所述至少一个第二值是来自所述信号的第二分量的值，其中，当每个所述探针仅产生一个第二值时，所述试剂盒能够检测在单一体积中的、以存在或不存在的任意组合形式的至少6个分析物中的每个分析物的存在或不存在。

[0049] 引用并入

[0050] 在本说明书中提到的所有的出版物和专利申请在此通过引用以相同程度并入，如同每个单独的出版物或专利申请被具体地和单独地表明通过引用并入。

附图说明

[0051] 本发明的新颖特征在所附权利要求书中特别陈述。将通过参考以下的详细描述的说明性实施方案的陈述获得对本发明的特征和优点更好地理解，其中本发明的原理被利用，且其中的附图是：

[0052] 图1显示了以四个颜色检测四个分析物的传统的编码方法，及以四个颜色能够检测16个或更多个序列的本发明的编码方法之间的比较。颜色由B、G、Y和R指示，其分别指示蓝色、绿色、黄色和红色。

[0053] 图2显示了表示以四个颜色对6个分析物(包括对照)检测的示意图。使用连接到荧光团(B、G、Y、R;分别为蓝色、绿色、黄色和红色)和猝灭剂(标有“X”的椭圆形)的杂交探针检测分析物。对照序列和五个其它序列(登革热＝登革热病毒;结核病＝结核分枝杆菌(Mycobacterium tuberculosis);P17＝HIV p17;疟疾＝恶性疟原虫(Plasmodium falciparum);和疱疹＝单纯性疱疹病毒2)均使用标有蓝色荧光团的探针检测。每个非对照分析物分别使用1-3个额外的探针检测。例如，登革热病毒的分析物使用三个具有绿色、黄色和红色荧光团的额外的探针检测;单纯性疱疹病毒2的分析物使用具有红色荧光团的额外的探针检测;等等。

[0054] 图3显示了如实施例2中描述得到的实验结果的色谱。

[0055] 图4显示了本发明的示例性的实施方案，其中使用计算机执行本文提供的方法的一个或更多个步骤。

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[0056] 图5显示了检测光信号，例如荧光发射信号的波长和强度的两个不同的方法的示意图。

[0057] 图6显示了示例性的试剂盒的示意图。

[0058] 图7显示了如实施例3中描述得到的实验结果的色谱。

[0059] 图8显示了合理的结果(上图)和不合理的结果(下图)的示意图。

具体实施方式

[0060] 由于几个理想的特性，包括与生化测定的相容性、荧光标记相对小的尺寸、缀合感兴趣的分子的简单的手段、可负担性、低毒性、稳定性、鲁棒性、利用廉价光学系统的可检测性、及与空间阵列相结合的能力，荧光检测已经是多路测定优选的技术。然而，标准的荧光具有广泛的发射光谱。因此，为了避免光谱重叠(即，为了保持光谱分辨率)仅相对少量数目的颜色(例如，4至6种)通常在多路荧光测定中同时使用。

[0061] 多路荧光测定的传统编码方法一直以单一的颜色编码每个分析物；即，M＝N，其中M是可检测的分析物的数目，且N是光谱可分辨的荧光探针的数目。每当需要更高的多路因子时(即，M>N)，荧光通常结合其它技术技术，如等分、空间阵列、或顺序处理。这些额外的处理步骤是劳动密集的，并经常需要相对昂贵和复杂的光学和机械系统(例如，光谱仪、机械化显微镜镜台、微流体、液滴生成器、扫描仪、及类似系统)。这种系统在某些环境中配置往往是不切实际的，特别是定点护理(point-of care)和低资源设置。因此，对可提供廉价的多路的手段，同时避免使用昂贵的额外的处理步骤的多路编码和解码方法有显著需求。

[0062] 本公开提供了用于样品中的多个分析物的检测的方法、系统、组合物和试剂盒。本文呈现了基于编码、分析和解码方法对分析物进行检测。在一些实例中，待检测的每个分析物被编码为信号(例如，强度)的值，其中值被分配，使得测定的结果明确地指示被测定的分析物存在或不存在。在其它实例中，待检测的每个分析物被编码为在信号的至少两个分量(例如，强度和波长)的每个中的值。信号的至少两个分量可以是正交的。类似地，如本公开中在其他地方更全面地描述的，可以使用多个正交信号，例如荧光信号和电化学信号的组合。分析物可以是任何合适的分析物，例如多核苷酸、蛋白质、小分子、脂质、碳水化合物、或其混合物。信号可以是任何合适的信号，例如电磁信号、光信号、荧光发射信号、电化学信号、化学发光信号、及其组合。信号的至少两个分量可以是任何合适的两个分量，例如振幅和频率，或强度和波长。

[0063] 编码分析物之后，样品被提供，其中样品包括或可包括编码的分析物中的至少一个。样品与在编码方案中对每个分析物指定的在分析物存在时、产生特定的信号的、一个或多个分析物特异性试剂接触。试剂可以是任何合适的试剂，其能够在其相应的分析物存在时产生这样的信号，例如，连接到荧光团和猝灭剂的寡核苷酸探针(例如，TAQMAN探针)。如果试剂是连接到荧光团和猝灭剂的寡核苷酸探针，可执行产生信号的核酸扩增。

[0064] 添加试剂后，信号被定量。在一些情况下，通过测量信号的一个分量(例如，荧光强度)，及基于用于编码每种分析物的存在的值、及信号的累积值，确定某些分析物存在和不存在，执行这种定量。

[0065] 在一些情况下，对样品累积地测量信号的至少两个分量(例如，强度和波长)。例如，这种测量可以通过测量在特定波长的强度，或特定波长范围内的强度执行。然后可以基

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于信号的至少两个分量中的每个分量的值与用于编码分析物的存在的值(即,在编码方案中的那些值)确定分析物的存在或不存在。

[0066] 编码可以以减少或消除通过该方法获得的可能的简并(例如,不明确的)结果的数目的方式执行。如本说明书中其他地方所描述的,特定编码方案的完整编码能力可被枚举,且某些潜在的分析物代码可从编码方案消除,以减少或消除简并性。同样地,编码方案可被设计成非简并的,使得简并性的减少或消除是没有必要的。可以构建解码矩阵以对应于累积信号测量结果的构成信号,将累积信号测量结果(例如,强度或在特定波长的强度)解释为某些分析物存在或不存在。

[0067] I.定义

[0068] 本文所用的术语仅用于描述特定实施方案的目的,并不旨在限制。

[0069] 如本文所用,单数形式的"一(a)"、"一(an)"、和"该(the)"除非上下文另有明确地指示,也旨在包括复数形式。此外,针对术语"包括(including)"、"包括(includes)"、"具有(having)"、"具有(has)"、"有(with)"、"例如(such as)"、或其变体的范围,在说明书和/或权利要求书中使用时,这些术语不是限制的,且旨在以类似术语"包括(comprising)"的方式被包括。

[0070] 如本文所用的术语"约",一般是指比特定用法的上下文中规定的数值增加15%或减少15%的范围。例如,"约10"将包括从8.5到11.5的范围。

[0071] 如本文所用的术语"维"和"分量",当涉及信号时,一般是指可被定量的信号的方面。例如,如果是由荧光分子产生的信号,其可以依据其波长(例如,第一维或第一分量)及其强度(例如,第二维或第二分量)被量化。

[0072] 如本文所用的术语"解码",一般是指确定哪个分析物存在的方法,其基于累积信号和能够将累积信号转换为关于一个或更多个分析物存在或不存在的信息的编码方案或解码矩阵。

[0073] 如本文所用的术语"编码",一般是指表示分析物的处理,使用包括信号的值(例如强度)、或在一个信号或多个信号的至少两个分量中的每个分量的值(如波长和强度)的代码。

[0074] 术语"连接到荧光团和猝灭剂的寡核苷酸探针"和"TAQMAN探针"通常是指在多核苷酸扩增测定中用于检测分析物存在的水解探针。这些探针包括连接到荧光团和猝灭剂的寡核苷酸探针。只要猝灭剂和荧光团接近,猝灭剂就会猝灭由光源激发荧光团所发射的荧光。寡核苷酸探针的序列被设计为互补于分析物中存在的多核苷酸序列,并因此,能够与分析物中存在的多核苷酸序列杂交。寡核苷酸探针的杂交在包括引物的核酸扩增反应(例如,聚合酶链式反应)中执行。通过DNA聚合酶延伸引物后,聚合酶的5'至3'外切酶活性降解探针,释放荧光团和猝灭剂至介质中。荧光团和猝灭剂之间的接近被破坏,且来自荧光团的信号不再猝灭。因此,检测到的荧光的量是分析物存在的量的函数。如果没有分析物存在,探针将不与分析物杂交,且荧光团和猝灭剂将保持极为接近。很少信号或没有信号会产生。

[0075] 如本文所用的术语"正交",一般是指可以独立地或近似独立地改变的信号的至少两个分量(如,波长和强度),或至少两个不同的信号(如,荧光发射信号和电化学信号)。例如,当使用荧光分子时,波长和强度被认为是正交的或近似正交的。除其它因素外,荧光发射的波长将取决于荧光分子的组合物,并且荧光的强度将取决于存在的分子的量。虽然波

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长和强度是可以近似独立地被改变的信号的两个分量的实例，但是本文描述的方法不限于可以独立地或近似独立地被改变的分量。也可以使用非独立地改变的信号的分量、或多个信号，只要足够良好地确定分量或信号的特征，以能够编码、测量和解码。例如，如果一个分量或信号中的变化影响另一个分量或信号中的变化，只要理解变化之间的关系，仍可以使用这两个分量或信号。

[0076] 如本文所用的术语“多核苷酸”、“寡核苷酸”、或“核酸”，在本文中用于指包括多个核苷酸的生物分子。示例性的多核苷酸包括脱氧核糖核酸、核糖核酸、及其合成的类似物，包括肽核酸。

[0077] 如本文所用的术语“探针”一般是指在特定的分析物存在下能够产生信号的试剂。探针通常具有至少两个部分：能够特异性识别分析物，或其一部分的部分；及能够在分析物，或其一部分存在下，产生信号的部分。如上文和本公开中其他地方描述的，探针可以是连接到荧光团和猝灭剂的寡核苷酸探针。探针还可以是在分析物存在下，产生信号的任何试剂，如检测分析物的抗体，具有因抗体结合至分析物而发射或被猝灭的荧光标记。任何合适的探针可以以本公开中提出的方法使用，只要探针在分析物存在下产生可定量的信号。例如，探针的分析物特异性部分可以与酶耦合，在分析物存在下，所述酶转化不带电荷的底物为带电荷的产物，从而随着时间的推移提高了介质中的导电率。在此情况下，不同的分析物可以以不同比例通过耦合探针(如杂交探针或抗体)的分析物特异性部分至酶被编码。介质中增加的导电率的产生速率将对介质中存在的所有分析物累积。根据本文提供的方法，编码分析物能够将导电率测量结果转换为明确的(即，非简并的)的结果，提供了关于特定分析物存在或不存在的信息。类似地，探针可包括从底物产生化学发光产物的酶。化学发光的量可随后被用于编码特定分析物的存在。

[0078] II.编码和解码方法

[0079] A.传统荧光编码和解码方法

[0080] 确定分析物存在的常用的方法是使用4个光谱可分辨的荧光分子指示四个分析物的存在或不存在。图1的左手侧表示了这种方法的实例。图1的左手侧显示了编码方法，其中四个分析物(序列1、序列2、序列3和序列4)每个分别由单一颜色(分别为蓝色、绿色、黄色和红色)编码。颜色表示连接到还包括猝灭剂的寡核苷酸探针的荧光团。在图1的左手侧所示的系统中，有四种不同的寡核苷酸探针，每种包括单一的荧光团(蓝色、绿色、黄色、或红色)和猝灭剂。分析物的存在或不存在是基于在特定的颜色中信号的存在或不存在确定的。

[0081] 图1的左手侧的图表显示了包含两个分析物：序列1和序列3，的假定样品的强度对颜色。这些分析物的存在是基于蓝色信号(对应于序列1)和黄色信号(对应于序列3)的测量结果确定的。不存在序列2和序列4是通过不存在绿色和红色信号指示的。

[0082] 表1显示了这种编码方案的二进制格式的解释。每个分析物被编码为在荧光信号的两个分量中的每个分量的值：(1)颜色(也称为波长；或波长范围)和(2)的强度(由表中的数字指示)。例如，A(例如，序列1)具有蓝色的颜色和1的强度；B(例如，序列2)具有绿色的颜色和1的强度；等等。如本文所述的定量每个颜色范围内的信号的强度，例如，通过测量由带通滤波器确定的特定波长范围内的信号的强度。1000的结果指示仅分析物A存在；1100结果指示分析物A和B存在；等等。

[0083] 表1.具有四个探针的四个分析物的传统编码，每个探针具有单一颜色，且每个分

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析物一个探针。

[0084]

	蓝色	绿色	黄色	红色
A	1	0	0	0
B	0	1	0	0
C	0	0	1	0
D	0	0	0	1

[0085] B.每种分析物使用一种以上的颜色的编码方法

[0086] 上文描述的传统方法经受其由光谱可分辨的荧光团的数目限制的事实。更具体地，可检测的分析物的数目等于光谱可分辨的荧光团的数目。因此，分析物的数目仅可通过增加光谱可分辨的荧光团的数目增加。

[0087] 本公开提供了克服这种限制的方法。更具体地，在一些情况下，通过在编码过程中，利用信号的至少两个分量，本文描述的方法可用于检测每个荧光团一个以上的分析物。例如，使用本文提供的方法，每个荧光团可检测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、或50个分析物。在一些情况下，本文提供的方法可用于检测，每个荧光团可检测至少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、或50个分析物。在一些情况下，本文提供的方法可用于检测每个荧光团1.5-2、2-4、1.5-4、4-6、2-6、6-10个分析物。

[0088] 在一些情况下，本公开提供的方法可包括使用对照颜色。对照颜色可连接到一个或更多个探针，在样品中，所述探针结合阳性对照分析物，及待检测的每个分析物。如果在阳性对照分析物和待检测的每个分析物中出现相同的序列，也可以使用单一的对照探针。如果阳性对照分析物和待检测的每个分析物中不出现相同的序列，可以使用不同的探针，但每个探针仍可连接到对照颜色。

[0089] 例如，建立在上文描述的传统方法上，一种颜色(例如蓝色)可用于编码始终存在于样品中的对照分析物的存在。对照分析物可被加入到样品中，或者可以是固有地存在于样品中。剩余的颜色(例如，绿色、黄色、红色)可用于编码额外的分析物的存在。表2显示了一个这样的方法的实例。

[0090] 表2.以四个颜色且每个分析物最多两个颜色编码四个分析物(包括对照)。

[0091]

	蓝色	绿色	黄色	红色
对照(p)	1	0	0	0
A	1	0	0	1
B	1	0	1	0
C	1	1	0	0

[0092] 在表2中，对照组(p)的存在由1000的结果指示。对照和分析物A的存在由2001的结果指示。对照和所有其它三个分析物的存在由4111的结果指示。对照颜色(蓝色)提供了测定正常运行的指示。蓝色颜色的强度报告了测试样品中存在的分析物的数目。当然，任何颜

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色都可以被用于对照颜色。本领域技术的一名人员将认识到，某些实际考虑可能使其优选使用一种颜色而不使用另一种颜色作为对照颜色。例如，如果一种颜色在特定的光学系统(或荧光团系统)中被更好地检测，那么可能实际上优选使用该颜色作为对照颜色。

[0093] 表2中所示的编码方案使用一个对照颜色和一个额外的颜色(每个分析物最多2个探针)编码每个分析物。然而，如下所示，当每个分析物使用多达4个颜色时，可被编码的分析物的数目增加。表3显示了示例性的编码方案，其中每个分析物由最多4个颜色编码。表3所示的方案中，对照颜色(蓝色)在对照序列和其它七个分析物(A-G)的每个分析物的存在下产生。其它七个分析物的每个由一至三个额外的颜色的存在编码。颜色可能被不同的探针(例如，连接到荧光团和猝灭剂的寡核苷酸探针)或相同的探针(其可以具有多个荧光团和多个猝灭剂)所包含。

[0094] 表3.以四个颜色且每个分析物最多四个颜色编码八个分析物(包括对照)。

[0095]

	蓝色	绿色	黄色	红色
对照(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

[0096] 在表3中，对照和所有其它七个分析物的存在由8444的结果指示。三个分析物(A、B和C)由两个颜色编码。三个分析物(D、E和F)由三个颜色编码，且一个分析物(G)由四个颜色编码。对照(p)的存在由1000的结果指示。对照和分析物A的存在由2001的结果指示。对照和分析物G的存在由2111的结果指示。这些结果表达每个颜色中信号的累积强度。例如，结果2111在蓝色通道中具有2X的信号强度，而结果1000在蓝色通道中仅具有1X的信号强度。

[0097] 使用表3，依据颜色(蓝色、绿色、黄色、红色)和强度(0-8)，每个可能的累积测定结果可被枚举。表4显示了通过基于在表3中所示的编码方法，对每个可能的累积测定结果进行枚举，并且根据在样品中存在的分析物提供每个测定的相应的解码结果，产生“解码矩阵”。通过基于编码方案枚举每个可能的累积测定结果，及根据在样品中存在的(或不存在的)分析物提供每个测定的相应的解码结果，可以产生用于本文描述的任何编码方案的类似的解码矩阵。在一些情况下，如以下所述，一个或更多个分析物可从编码方案除去，以减少或消除简并性。

[0098] 表4.表3所示的编码方法的解码矩阵。

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[0099]

累积测定结果				
蓝色	绿色	黄色	红色	存在的分析物
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

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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
[0100] 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

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[0101]

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

[0102] 使用两个假定构建的表4中提供了解码矩阵。首先，解码矩阵假定阳性对照(P)总是产生阳性的结果。第二，解码矩阵假定，每个颜色中，无论信号可来自哪个探针，强度都以与改变探针浓度相同的方式添加和衡量。这本质上意味着信号是加性的和数字的。这些假定所依据的条件可以通过适当准备测定得到满足。如在本文所提供的实施例中证明的，如果使用荧光信号，强度仅需近似是的加性的和数字的。

[0103] 表4允许荧光波长的四个范围中强度(即，每个颜色范围中的信号强度)的累积测量转换为样品中存在的相应的分析物。例如，4321的结果指示存在p、C、F和G，且其它分析物不存在。此结果被称为“合理”的结果，因为其在解码矩阵中出现。相比之下，4000的结果，虽然可能测量到，但未在解码矩阵出现。更具体地，4000的结果不能通过添加来自表3的对照代码和分析物代码的任意组合被实现。该结果被称为“不合理”的结果。不合理的结果可能指示测定故障。因此，对照(p)和解码矩阵提供了验证测定是否正常运行的手段。

[0104] 表4穷举了产生4个可分辨发射光谱(例如，颜色)的荧光团的任意组合。术语“秩

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(rank)”用于描述检测的包括对照的分析物的数目。在上文提供的实例中，如果测定正常运行，那么秩等于蓝色信号的值。例如，秩8指示对照及所有其它七个分析物(A-G)存在。秩2指示存在对照及仅有的一个分析物。最低的秩是秩1，这指示仅存在对照。在每个秩的可能性的数目可被枚举。例如，继续参考在表3-4中描述的编码和解码方法，对于秩2有7个可能性。在秩3的可能性的数目可以以7取2的组合计算或者说有7!/(5!*2!)＝21个可能性。更普遍地，可能性的数目是N取K的组合，即为N!/((N-K)!*K!)。类似地，在秩4、5、6、7和8，可能性的数目分别是35、35、21、7和1。参照表4显示了表与理论预测一致。因此，表4是对表3中提供的编码方法的穷举的解码矩阵。

[0105] C.减少或消除简并性

[0106] 如本文使用的术语“简并”和“简并性”，通常描述合理的结果是不明确的情况，因为依据分析物存在或不存在，其可以指示一个以上的可能性。例如，参照表4，结果5233是简并的，因为它可以被解码为pADFG或pBDEG。类似地，结果4222是简并的，因为它可以被解码为以下任一个：pCDG、pAFG、pBEG、pDEF。相比之下，结果3110只能指示pBC，且因此是非简并的。

[0107] 本公开提供了减少或消除简并性的方法，从而提高了通过其检测到分析物的置信度。在一个实施方案中，消除简并度的方法包括：(i)将待检测的每个潜在分析物编码为信号的值，并且可选地，编码为信号的至少两个分量的每个分量中的值；(ii)枚举可以从编码方案获得的每个合理的结果；(iii)鉴定是简并的每个合理的结果；及(iv)从编码方案消除至少一个潜在分析物(或潜在的分析物代码)，其中消除该至少一个潜在的分析物减少或消除简并性。例如，参照表3中描述的编码方案，及在表4中描述的解码矩阵(枚举每个合理的结果)，消除分析物D、E和F的任意两个，消除了简并性。消除分析物D、E和F中的任意一个不会消除简并性，但会减少它。

[0108] 继续参照表3中描述的编码方案，从编码方案消除分析物D、E和F的任意两个，产生仅使用4个颜色，以无简并性可以分析6个分析物(包括对照)的方案。相比之下，多路常规方法使用4种颜色将仅允许报告3个分析物和1个对照。因此，通过使用本文提供的方法可被分析的分析物的数目接近加倍。

[0109] 图2显示了本发明的示例性实施方案，其中使用4个颜色检测五个分析物和一个对照。分析物是来自登革热病毒(“登革热”)、结核分枝杆菌(“结核病”)、人类免疫缺陷病毒(HIV)p17(P17)、疟原虫(“疟疾”)、和单纯性疱疹(“疱疹”)的核酸。在此实例中“颜色”是连接到还包括猝灭剂的寡核苷酸探针的荧光团。寡核苷酸探针通常将对不同的分析物不同，而颜色是相同的。例如，指定为探针1的所有探针具有蓝色颜色，但通常会具有不同的寡核苷酸序列。当然，如果互补序列在每个分析物中存在，指定为探针1的探针也可以具有相同的寡核苷酸序列。本公开中其他地方描述了用这些探针检测的机制。探针1(蓝色)杂交包括对照的所有6个分析物。探针2(绿色)杂交来自登革热病毒、分歧杆菌和HIV p17的分析物。探针3(黄色)杂交来自登革热病毒和疟原虫的分析物。探针4杂交来自登革热病毒、分歧杆菌和单纯性疱疹的分析物。通过这些探针定义的编码方案被示于表5。参照表3，潜在的分析物D(1011)和F(1110)已经从编码方案消除。因此，表5中所示的编码方案是非简并的，且来自测定的每个合理的结果对应于样品中分析物的唯一的组合的存在或不存在。

[0110] 表5.如图2所示例的，用于检测登革热病毒、分枝杆菌、HIV、疟原虫和单纯性疱疹

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的编码方案。

[0111]

	蓝色	绿色	黄色	红色
对照（p）	1	0	0	0
单纯性疱疹	1	0	0	1

[0112]

疟原虫	1	0	1	0
HIV	1	1	0	0
分歧杆菌	1	1	0	1
登革热病毒	1	1	1	1

[0113] 上文所示的用于编码和解码的方法，包括用于消除简并性的方法，都同样地适用于使用额外的颜色的编码方法。例如，表3可以通过包括额外的颜色和额外的强度扩大（下面进一步描述）。然后如上所述，枚举可以从编码方案获得的每个合理的结果产生解码矩阵。可以为本公开中描述的任何编码方案构建类似于表4中提供的解码矩阵的解码矩阵。然后鉴定简并的合理的结果，且至少一个潜在的分析物代码从编码方案消除，以减少或消除简并性。消除简并性的方法可以使用在计算机可读介质上的软件、或使用执行该方法的硬件配置（例如，微芯片）执行。

[0114] 虽然简并性可由以上、及在本公开中其他地方描述的方法减少或消除，本公开还提供了通过设计的非简并的编码方案。例如，消除表3中描述的编码方案的简并性后，编码方案可无限地以非简并的方式扩大，其中非简并性是通过设计（参见例如，表6中示例的编码方案，下文更充分地描述）。类似地，本公开提供通过设计的完全非简并的编码方案，且因此不需要任何简并性减少或消除（参见例如，表8中示例的编码方案，下文更充分地描述）。因此，本公开提供的编码方案可能已经通过设计减少或消除了简并性，或通过设计是非简并的。

[0115] D.使用一个以上的颜色和一个以上的强度的编码方法

[0116] 上文描述的编码方法（例如，表3-5）可以通过允许由大于1的强度编码分析物被进一步扩大。例如，表5中提供的编码方案中的编码的每个分析物是由1或0的荧光强度编码的。在至少一个颜色中允许较高的信号强度值进一步增加了可以通过本公开中提供的任何方法编码的分析物的数目。在一些实例中，为了保持对照颜色的分析物计数能力，这些较高的强度值可被分配除了对照颜色的任何颜色。

[0117] 表6显示了利用四个颜色和多强度的示例性编码方案的最前面的三层。表6的第1层是表3的复制，显示了使用四个颜色的七个分析物和一个对照的编码。如上文描述的，潜在的分析物D、E和F的任何两个可从编码方案消除，以便产生非简并的编码方案。表6的第1层指示潜在的分析物D（1011）和F（1110）已经从编码方案消除，以消除简并性。因此，如上文描述的，表6的第1层所示的编码方案能够使用四个颜色确定五个分析物及一个对照的存在。

[0118] 表6的第1层的编码方案可通过允许任何颜色的强度增加，被扩大到第二层（层2）。如上文描述的，为了保持对照的序列计数能力，对照颜色的强度可保持为1。增加任何剩余三个颜色的强度将产生代码100Y、10Y0、1Y00、10YY、1Y0Y、1YY0和1YYY，其中Y>1。用于编码新的层的Y的最小值等于来自现有层的累积最大值加1。如下文描述的，也可以使用大于1的

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值，以最大化强度之间的差异。

[0119] 因此，在此上下文中，术语“层”通常用于描述充分利用通过特定数目的没有简并性的第一值(例如，强度)和第二值(例如，颜色)，提供编码能力的代码组。例如，表6的第1层充分利用通过没有简并性的四个颜色与每个颜色最多1个强度提供的编码能力。如表6所示，第1层，这将导致包括对照的六个编码的分析物。引入第二层，如上所述，Y的最小值可被增加至等于根据现有层的累积最大结果加1(或多于一)。在表6提供的实例中，蓝色(对照)颜色的强度被保持为1，以保持这种颜色的序列计数能力。因此，第2层包括五个非简并的编码的可能性，其通过将绿色、黄色和红色通道中的强度增加至等于根据第1层的这些通道的每个通道中的累积最大结果加一获得。然后可以枚举层2的这些代码的所有可能性，且导致简并性的代码可被消除，或第2层可以通过设计，使用用于消除来自第1层的对应的代码的信息，制成非简并的。然后可通过添加根据类似的方法构建的第三层、或更多的层实现更大的编码容量。编码方案可具有无限数目的层，例如1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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或更多层。

[0120] 更具体地，参照表6，第2层中的分析物H由1004编码。对照颜色的值保持为1。红色颜色的值等于根据先前层的累积最大结果(3)加1，或4。同样地，分析物I和J由1030和1400分别编码。这些代码的组合用于编码其余四个分析物(K-N)，如在第1层对分析物D-G完成的。这样完成了第2层。在第2层，潜在的分析物K(1034)和M(1430)的内容导致简并性。因此，这些分析物已从编码方案消除，以消除简并性。

[0121] 继续参照表6，第三层(层3)使用上文描述的相同的原理构建。分析物O用1-0-0-16编码。对照颜色的值仍然保持为1。红色颜色的值等于根据先前层的累积最大结果(15)加1、或16。同样地，分析物P和Q分别由1-0-9-0和1-16-0-0编码。这些代码的组合用于编码其余四个分析物(R-U)，如在第1层对分析物D-G及在第2层对分析物K-N完成的。在第3层，潜在的分析物R(1-0-9-16)和T(1-16-9-0)的内容导致简并性。因此，这些分析物已从编码方案消除，以消除简并性。

[0122] 表6中显示的三层的编码方案显示了使用四个颜色的15个分析物和一个对照的编码。通过增加更多的强度以产生额外的层和/或增加更多的颜色以在层内产生额外的编码能力，此编码方案可无限地扩大。如在本公开中描述的，减少或消除简并性的方法可以在添加每个强度和/或颜色时使用，以减少或消除简并的结果。

[0123] 更普遍地，表6中描绘的编码方案是表3-5中描绘的编码方案的非简并的、无限扩大。在第一层的累积测量的最大强度是6。在第二层的累积测量的最大强度是15。在第三层的累积测量的最大强度是63，且以此类推。给出最大累积强度值(F)，本编码方案中可用的层(T)的最大数目为$T=\log_4(F+1)$。表6中描绘的编码方案提供了每层五个(P个)非简并的代码。因此，代码的最大数目$M=5*\mathrm{Log}_4(F+1)$，或$M=P*T$，其中，P为每层的非简并的代码的数目，且T是层数。例如，给出$F=63$，对每层5个非简并的化码，代码(即，分析物)的最大数目为15。此式不包括代码1000，其在表6中保留为阳性对照。为了在分析物的总数中包括对照，任何人可简单地增加一，提供公式$M=(P*T)+1$。

[0124] 本公开中提供的方法可用于无限扩大此编码方案。例如，通过利用不同的强度(即，第一值)和颜色(即，第二值)的组合，任何人可以通过改变每层的非简并的代码数目

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(P)和层的数目(T)，编码任何数目(M)的分析物。例如，每层的非简并的代码的数目是1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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或更多。类似地，层的数目可以是1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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或更多。

[0125] 表6.使用四个颜色和多个强度编码15种分析物和1个对照。

层	分析物	B	G	Y	R	备注
1	对照（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~~	从编码方案中消除以消除简并性
	E	1	1	0	1	
	~~F~~	~~1~~	~~1~~	~~1~~	~~0~~	从编码方案中消除以消除简并性
	G	1	1	1	1	
累积最大结果		6	3	2	3	
2	H	1	0	0	4	
	I	1	0	3	0	
	J	1	4	0	0	
	~~K~~	~~1~~	~~0~~	~~3~~	4	从编码方案中消除以消除简并性
	L	1	4	0	4	
	~~M~~	~~1~~	4	~~3~~	~~0~~	从编码方案中消除以消除简并性
	N	1	4	3	4	
累积最大结果		11	15	8	15	
	O	1	0	0	16	
	P	1	0	9	0	
	Q	1	16	0	0	
	~~R~~	~~1~~	~~0~~	~~9~~	~~16~~	从编码方案中消除以消除简并性

[0126]

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[0127]

	S	1	16	0	16	
	~~T~~	~~1~~	~~16~~	~~9~~	~~9~~	从编码方案中消除以消除简并性
	U	1	16	9	16	
累积最大结果						
3个非简并层的累积最大结果		**16**	**63**	**26**	**63**	

[0128] 图1的右手侧显示了上文描述的方法的一个示例性的实例。更具体地，图1的右手侧显示了编码方法，其中9个或多于9个分析物(序列1-序列9，等)中的每种个由至少两个颜色，且在每个颜色的强度变化进行编码。颜色表示连接到还包括猝灭剂的寡核苷酸探针的荧光团。系统可设计成使得每个探针以单一荧光团标记，或每个探针以一个以上的荧光团标记。例如，对于分析物H的代码，在表6中是1004。在红色通道中4的强度可通过使用包括4个红色荧光团的H特异性探针，或者通过使用每个包括单一的红色荧光团的4个H特异性探针实现。当然，产生在红色通道中4的结果的探针和荧光团的任意组合也同样合适，如每个含有2个红色荧光团的2个探针，及含有1个红色荧光团的1个探针和含有3个红色荧光团的1个探针，或仅仅是含有一个红色荧光团的一个探针，但以4x数量在反应混合物中存在。

[0129] 图1的右手侧描绘的编码方案可以如表7中所示。图1的右手侧所示的分析结果是4112，或蓝色通道中强度为4、绿色通道中为1、黄色通道中为1、且红色通道中为2。使用如本文所述构建的解码矩阵，此结果被解码为指示序序列1、序列3、序列4和序列5存在。

[0130] 表7.图1的右手侧说明的编码方案。

[0131]

层	分析物	B	G	Y	R	备注
1	**序列 1**	1	0	0	0	
	序列 2	1	1	0	0	
	序列 3	1	0	1	0	
	序列 4	1	0	0	1	
	序列 5	1	1	0	1	
	序列 6	1	1	1	1	

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[0132]

	~~消除的潜在的序列~~	~~1~~	~~0~~	~~1~~	~~1~~	从编码方案中消除以消除简并性
	~~消除的潜在的序列~~	~~1~~	~~1~~	~~1~~	0	从编码方案中消除以消除简并性
累积最大结果		6	3	2	3	
2	**序列 7**	1	4	0	0	
	序列 8	1	0	3	0	
	序列 9	1	0	0	4	
等等-即，以如在表 6 中提供的继续						

[0133] E.每个分析物使用一个颜色和一个强度，但在分析物中使用不同强度的编码方法

[0134] 在本文提供的一些方法中，每个分析物是由单一的颜色和强度的组合编码的。例如，在四色系统中，最前面的四个分析物可通过1000、2000、4000和8000编码。其后的四个分析物可通过0100、0200、0400和0800编码。分析物9-12和13-16将类似地分配，如表8所示。

[0135] 像表6中描述的编码方法，该编码方案通过构建在理论上是无限的、非简并的，且仅由测量信号使用的仪器的带宽限制。然而，此编码方案比表6中描述的编码方法能够在每单位带宽上定量更多的分析物。其原因在于，以最大的效率使用信号的可用的多路性，因为编码中利用了每个强度等级(即色谱中没有空缺；参见下文色谱的描述)。表8显示了该方法的一个实施方案，说明了基于四个颜色和强度1、2、4和8的四层编码。编码方案是非简并的，且如果所有16个分析物存在，结果是15-15-15-15。在带宽利用率方面，这种编码方法比上面给出的编码方法更有效。然而，这种方法不具有第一个方案的校对能力，这是因为第一个方案中所有解码为合理的输出结果的结果都存在色谱中的空缺。

[0136] 表8.每个分析物使用一个颜色和一个强度，但在分析物中使用不同强度的编码方法的实例。

[0137]

层	分析物	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

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[0138]

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

[0139] 上文所示的，及表8中示例的方法，可通过引入额外的颜色和/或强度扩大。例如，可以使用5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个颜色。也可以使用额外的强度等级，例如16、32、64、128、256、512、1024、2048、4096、8192、16384、32768、65536、等等。该方法是可归纳的且可用于编码无限数目的分析物。每个分析物由单一颜色中的代码表示，其中，在该单一颜色中的代码的值等于所有先前值的总和加1。例如，如果在特定的颜色中的第一代码包含1，其后的代码是2、4、8、16、32、64、128等等。其它级数(progression)是可能的，但依据带宽的使用不会被认为有效的。然而，本领域的一个普通技术人员将认识到，当任何人希望最大化值之间的间隔时，可能希望较不有效的带宽使用。类似地，值1可从编码方案排除，例如，以最大化第一编码值和仪器的噪声之间的强度中的差异。例如，在一些情况下，编码方案可以以值2开始，其将为分析物代码提供为2、4、8、16、32、64、128、等等的级数，而累积结果的级数将是2、4、6、8、10、12、14、16、等等。这种方法在可能的累积结果中产生均匀的空缺(gap)，相比1、2、4、8、16、32、等等的分析物代码级数，允许对噪声更高的耐受，且其对应于1、2、3、4、5、6、7、8、等等的累积结果级数。在这个实例中，测量中增加的鲁棒性以多路性的固定带宽中可用的代码的数目减少为代价。例如，如果仅有63个多路性状态能够被可靠地测量，那么从1开始的编码方案每个颜色提供7个代码，而从2开始的编码方案每个颜色提供6个代码。如果4个颜色可用，前者将提供28个代码，而后者将提供24个代码。总之，开始信号强度和级数(即，强度值之间的差异)两者均可以被衡量，以最大化对仪器的噪声的分辨能力，并最大化强度值之间的差异，由此增强区分不同实验结果之间的能力。

[0140] 如表8说明的编码方案通过设计为非简并的。虽然表8中说明的编码方案使用强度

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和颜色两者编码16个分析物的每个分析物，还可以通过简单地利用强度编码每个分析物。例如，给出表8中提供的16个分析物(A-P)，单一颜色的编码方案编码全部16个分析物，可分别地分配值1、2、4、8、16、32、64、128、256、512、1024、2048、4096、8192和16384至分析物A、B、C、D、E、F、G、H、I、J、K、L、M、N、O和P。这种编码方案通过设计为非简并的，且累积强度结果可以被明确地解码以指示分析物A-P的存在或不存在。这种编码方案能够检测M种分析物的存在或不存在，其中$M=Log_2(F+1)$，且F是信号(例如，信号强度)的最大累积值。通过向这个信号添加第二分量(如颜色，如表8中描绘的)，这个编码方案的容量可以增大到$M=C*Log_2(F+1)$，其中C是在所述编码方案中使用的颜色数目。

[0141] 如贯穿此说明书中描述的，任何合适的值可以用于C或F。例如，C可以是至少约2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多。F+1可以是至少约2、4、8、16、32、64、128、256、512、1024、2048、4096、8192、16384、32768、65536、131072、262144、524288、1048576、等等。如在本公开中其他地方描述的，F的最初开始值和在其级数中的步长(step)也可以改变。

[0142] III.分析物

[0143] 分析物可以是使用本发明的方法和组合物分析的任何合适的分析物，其中分析物能够与试剂相互作用，以便产生可被测量的具有至少两个分量的信号。分析物可以是天然存在的或合成的。分析物可以存在于使用本领域中已知的任何方法获得的样品中。在一些情况下，样品可以在其用于分析物的分析前被处理。本公开中提出的方法和组合物可在溶液相测定中使用，而不需要颗粒(如珠)或固体支持体。

[0144] 在一些情况下，分析物可以是多核苷酸，例如DNA、RNA、肽核酸、及其任何杂合体(hybrid)，其中多核苷酸包含脱氧核糖核苷酸和/或核糖核苷酸的任意组合。多核苷酸可以是单链或双链的，或同时含有双链或单链序列部分。多核苷酸可包含核苷酸或碱基的任意组合，包括，例如，尿嘧啶、腺嘌呤、胸腺嘧啶、胞嘧啶、鸟嘌呤、肌苷、黄嘌呤、次黄嘌呤、异胞嘧啶、异鸟嘌呤及其任意核苷酸衍生物。如本文所用，术语“核苷酸”可包括核苷酸和核苷，以及核苷和核苷酸类似物，及修饰(modified)的核苷酸，包括合成的种类和天然存在的种类两者。多核苷酸可以是针对其可产生如本文描述的一种或更多种试剂(或探针)的任何合适的多核苷酸，包括但不限于cDNA、线粒体DNA(mtDNA)、信使RNA(mRNA)、核糖体RNA(rRNA)、转移RNA(tRNA)、核RNA(nRNA)、小干扰RNA(siRNA)、小核RNA(snRNA)、小核仁RNA(snoRNA)、小卡哈尔体特异性RNA(small Cajal body-specific RNA，scaRNA)、微小RNA(miRNA)、双链(dsRNA)、核酶、核糖开关(riboswitch)或病毒RNA。多核苷酸可被包含在任何合适的载体中，例如质粒、粘粒、片段、染色体、或基因组。

[0145] 基因组DNA可从天然存在的或遗传修饰的生物体，或从人工地或合成地产生的基因组获得。包括基因组DNA单独分析物可从任何来源，且使用本领域中已知的任何方法获得。例如，基因组DNA可以利用或不利用扩增被分离。扩增可包括PCR扩增、多重置换扩增(MDA)、滚环扩增和其它扩增方法。基因组DNA也可以通过克隆或重组的方法获得，例如那些涉及质粒和人工染色体的方法或其它的常规方法(参见Sambrook和Russell，Molecular Cloning:A Laboratory Manual.，出处同上)。多核苷酸可使用本领域已知的其它方法分离，例如如在Genome Analysis:A Laboratory Manual Series(卷I-IV)或Molecular

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Cloning:A Laboratory Manual中公开的。如果分离的多核苷酸是mRNA,其可以使用常规技术被逆转录(reverse transcribe)为cDNA,如Sambrook和Russell,Molecular Cloning:A Laboratory Manual,出处同上,中描述的。

[0146] 分析物可以是蛋白质、多肽、脂质、碳水化合物、糖、小分子、或可以以本文提供的方法和组合物检测的任何其它合适的分子。分析物可以是酶或其它蛋白质。分析物可以是药物或代谢物(例如,抗癌药物、化学治疗药物、抗病毒药物、抗生素药物、或生物制剂(biologic))。分析物可以是任何分子,如辅因子、受体、受体配体、激素、细胞因子、血液因子、抗原、类固醇、或抗体。

[0147] 分析物可以是来自任何病原体的任何分子,病原体例如病毒、细菌、寄生虫、真菌、或朊病毒(例如,PrP^{Sc})。病毒的实例包括来自以下科的那些,腺病毒科、黄病毒科、嗜肝病毒科、疱疹病毒科、正粘病毒科、乳多空病毒科、副粘病毒科、小核糖核酸病毒科、多瘤病毒科、逆转录病毒科、弹状病毒科、及披膜病毒科。病毒的具体实例包括腺病毒、星状病毒、博卡病毒、BK病毒、柯萨奇病毒、巨细胞病毒、登革热病毒、埃博拉病毒、肠病毒、EB病毒、猫白血病病毒、肝炎病毒、甲型肝炎病毒、乙型肝炎病毒、丙型肝炎病毒、丁型肝炎病毒、戊型肝炎病毒、单纯性疱疹病毒(HSV)、HSV 1型、HSV 2型、人免疫缺陷病毒(HIV)、HIV 1型、HIV 2型、人类乳头瘤病毒(HPV)、HPV 1型、HPV 2型、HPV 3型、HPV 4型、HPV 6型、HPV 10型、HPV 11型、HPV16型、HPV18型、HPV 26型、HPV 27型、HPV 28型、HPV 29型、HPV 30型、HPV 31型、HPV 33型、HPV 34型、HPV 35型、HPV 39型、HPV 40型、HPV 41型、HPV 42型、HPV 43型、HPV 44型、HPV 45型、HPV 49型、HPV 51型、HPV 52型、HPV 54型、HPV 55型、HPV 56型、HPV 57型、HPV 58型、HPV 59型、HPV 68型、HPV 69型、流感病毒、JC病毒、马尔堡病毒、麻疹病毒、腮腺炎病毒、诺沃克病毒、细小病毒、脊髓灰质炎病毒、狂犬病病毒、呼吸道合胞病毒、逆转录病毒、鼻病毒、轮状病毒、风疹病毒、天花病毒、牛痘病毒、西尼罗河病毒和黄热病病毒。

[0148] 细菌的实例包括来自以下属的那些,博德特氏菌属(Bordetella)、疏螺旋体属(Borrelia)、布鲁氏菌属(Brucella)、弯曲杆菌属(Campylobacter)、衣原体属(Chlamydia)、梭菌属(Clostridium)、棒状杆菌属(Corynebacterium)、肠球菌属(Enterococcus)、埃希氏菌属(Escherichia)、弗朗西斯氏菌属(Francisella)、嗜血杆菌属(Haemophilus)、螺杆菌属(Helicobacter)、军团菌属(Legionella)、钩端螺旋体属(Leptospira)、李斯特菌属(Listeria)、分枝杆菌属(Mycobacterium)、支原体属(Mycoplasma)、奈瑟菌属(Neisseria)、假单胞菌属(Pseudomonas)、立克次氏体属(Rickettsia)、沙门氏菌属(Salmonella)、志贺氏菌属(Shigella)、葡萄球菌属(Staphylococcus)、链球菌属(Streptococcus)、密螺旋体属(Treponema)、弧菌属(Vibrio)和耶尔森氏菌属(Yersinia)。细菌的具体实例包括副百日咳博德特氏菌(Bordetella parapertussis)、百日咳博得特氏菌(Bordetella pertussis)、伯氏疏螺旋体(Borrelia burgdorferi)、流产布鲁氏菌(Brucella abortus)、犬布鲁氏菌(Brucella canis)、羊布鲁氏菌(Brucella melitensis)、猪布鲁氏菌(Brucella suis)、空肠弯曲杆菌(Campylobacter jejuni)、肺炎衣原体(Chlamydia pneumoniae)、鹦鹉热衣原体(Chlamydia psittaci)、沙眼衣原体(Chlamydia trachomatix)、肉毒梭菌(Clostridium botulinum)、艰难梭菌(Clostridium difficile)、产气荚膜梭菌(Clostridium perfringens)、破伤风梭菌(Clostridium tetani)、白喉棒状杆菌(Corynebacterium

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diphtheriae)、粪肠球菌(Enterococcus faecalis)、屎肠球菌(Enterococcus faecium)、大肠杆菌(Escherichia coli)、土拉弗朗西斯菌(Francisella tularensis)、流感嗜血杆菌(Haemophilus influenzae)、幽门螺旋杆菌(Helicobacter pylori)、嗜肺军团菌(Legionella pneumophila)、问号钩端螺旋体(Leptospira interrogans)、单增李斯特菌(Listeria monocytogenes)、麻风分枝杆菌(Mycobacterium leprae)、结核分枝杆菌(Mycobacterium tuberculosis)、溃疡分枝杆菌(Mycobacterium ulcerans)、肺炎支原体(Mycoplasma pneumoniae)、淋球奈瑟菌(Neisseria gonorrhoeae)、脑膜炎奈瑟菌(Neisseria meningitidis)、铜绿假单胞菌(Pseudomonas aeruginosa)、立克次氏体(Rickettsia rickettsii)、猪霍乱沙门氏菌(Salmonella choleraesuis)、都柏林沙门氏菌(Salmonella dublin)、肠炎沙门氏菌(Salmonella enteritidis)、伤寒沙门氏菌(Salmonella typhi)、鼠伤寒沙门氏菌(Salmonella typhimurium)、宋内氏志贺菌(Shigella sonnei)、金黄色葡萄球菌(Staphylococcus aureus)、表皮葡萄球菌(Staphylococcus epidermidis)、腐生葡萄球菌(Staphylococcus saprophyticus)、无乳链球菌(Streptococcus agalactiae)、肺炎链球菌(Streptococcus pneumoniae)、化脓性链球菌(Streptococcus pyogenes)、苍白密螺旋体(Treponema pallidum)、霍乱弧菌(Vibrio cholerae)、鼠疫耶尔森氏菌(Yersinia pestis)和小肠结肠炎耶尔森菌(Yersinia enterocolitica)。

[0149] 寄生虫的实例包括来自以下属的那些,棘阿米巴属(Acanthamoeba)、巴贝虫属(Babesia)、巴拉姆西属(Balamuthia)、肠袋属虫(Balantidium)、芽囊原虫属(Blastocystis)、隐孢子虫属(Cryptosporidium)、双核阿米巴属(Dientamoeba)、内阿米巴属(Entamoeba)、贾第虫属(Giardia)、等孢球虫属(Isospora)、利什曼原虫属(Leishmania)、耐格里虫属(Naegleria)、虱属(Pediculus)、疟原虫属(Plasmodium,)、鼻孢子虫属(Rhinosporidium,)、肉孢子虫属(Sarcocystis)、血吸虫属(Schistosoma)、弓形虫属(Toxoplasma)、滴虫属(Trichomonas)和锥虫属(Trypanosoma)。寄生虫的具体实例包括分歧巴贝虫(Babesia divergens)、双芽巴贝虫(Babesia bigemina)、马巴贝虫(Babesia equi)、田鼠巴贝虫(Babesia microti)、邓肯巴贝虫(Babesia duncani)、狒狒巴拉姆西阿米巴(Balamuthia mandrillaris)、结肠小袋纤毛虫(Balantidium coli)、脆弱双核阿米巴(Dientamoeba fragilis)、痢疾阿米巴(Entamoeba histolytica)、贾第鞭毛虫(Giardia lamblia)、贝利等孢球虫(Isospora belli)、福氏耐格里阿米巴(Naegleria fowleri)、人虱(Pediculus humanus)、恶性疟原虫(Plasmodium falciparum)、诺氏疟原虫(Plasmodium knowlesi)、三日疟原虫(Plasmodium malariae)、卵形疟原虫(Plasmodium ovale)、间日疟原虫(Plasmodium vivax)、鼻孢子虫(Rhinosporidium seeberi)、牛人肉孢子虫(Sarcocystis bovihominis)、猪人肉孢子虫(Sarcocystis suihominis)、曼氏血吸虫(Schistosoma mansoni)、弓形虫(Toxoplasma gondii)、阴道毛滴虫(Trichomonas vaginalis)、布氏锥虫(Trypanosoma brucei)、和克氏锥虫(Trypanosoma cruzi)。

[0150] 真菌的实例包括来自以下属的那些,鳞质霉属(Apophysomyces)、曲霉属(Aspergillus)、芽生菌属(Blastomyces)、念珠菌属(Candida)、枝孢霉属(Cladosporium)、球孢子菌属(Coccidioides)、隐球菌属(Cryptococcus)、突脐孢属(Exserohilum)、镰刀菌属(Fusarium)、组织胞浆菌属(Histoplasma)、毕赤酵母属(Pichia)、肺囊虫属

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(Pneumocystis)、酵母属(Saccharomyces)、孢子丝菌属(Sporothrix)、葡萄穗霉属(Stachybotrys)和毛癣菌属(Trichophyton)。真菌的具体实例包括烟曲霉(Aspergillus fumigatus)、黄曲霉(Aspergillus flavus)、棒曲霉(Aspergillus clavatus)、皮炎芽生菌(Blastomyces dermatitidis)、白色念珠菌(Candida albicans)、粗球孢子菌(Coccidioides immitis)、新型隐球菌(Cryptococcus neoformans)、尖角突脐孢(Exserohilum rostratum)、轮状镰刀菌(Fusarium verticillioides)、荚膜组织胞浆菌(Histoplasma capsulatum)、杰氏肺囊虫(Pneumocystis jirovecii)、申克孢子丝菌(Sporothrix schenckii)、黑葡萄穗霉(Stachybotrys chartarum)和须癣毛癣菌(Trichophyton mentagrophytes)。

[0151] 在一些情况下,本公开中所提供的方法可以用于检测在上文、或者在说明书的其它地方描述的分析物中的任何一个。在一些情况下,本公开中提供的方法可以用于检测在上文、或在说明书的其它地方描述的分析物的组合(panel)。例如,组合可包括选自由任意2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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或更多个在上文或在说明书中的其它地方描述的分析物所组成的组的分析物。

[0152] 分析物可从任何合适的位置获得,包括从生物体、完整细胞、细胞制剂和来自任何生物体、组织、细胞、或环境的无细胞组合物。分析物可从环境样品、活检、抽吸物、福尔马林固定的包埋组织、空气、农业样品、土壤样品、石油样品、水样、或灰尘样品中获得。在一些情况下,分析物可以从体液获得,其可能包括血液、尿液、粪便、血清、淋巴液、唾液、粘膜分泌物、汗液、中枢神经系统流体、阴道液、或精液。分析物也可以由制造的产品获得,如化妆品、食品、个人护理产品、等等。分析物可以是实验操作的产物,包括重组克隆、多核苷酸扩增、聚合酶链式反应(PCR)扩增、纯化方法(如基因组DNA或RNA的纯化)和合成反应。

[0153] 在每个多路测定中,可以检测多于一种类型的分析物。例如,多核苷酸、蛋白质、多肽、脂质、碳水化合物、糖、小分子、或任何其它合适的分子可以同时在相同的多路测定中以合适的试剂检测。可在相同的时间检测分析物的任意组合。

[0154] 分析物的检测可对任何合适的应用有用,应用包括科研、临床、诊断、预后、法医和监测应用。典型应用包括遗传性疾病的检测、遗传指纹的鉴定、感染性疾病的诊断、基因的克隆、亲子测试、刑事鉴定、系统发育、生物反恐、环境监测、及DNA计算。例如,分析物可以指示疾病或状况。分析物可以用来做出治疗决定,或以评估疾病的状态。分析物的存在可以指示具有特定病原体感染或任何其它疾病如癌症、自身免疫性疾病、心肺病、肝病、消化道疾病、等等。因此本文提供的方法可以用于做出诊断并基于该诊断做出临床决定。例如,指示从受试者采集的样品中细菌多核苷酸的存在的结果可能导致以抗生素治疗受试者。

[0155] 在一些情况下,本发明的方法和组合物可用于检测至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或1000个分析物。在一些情况下,本发明的方法和组合物可用于检测7-50、8-40、9-30、10-20、10-15、8-12、或7-12个分析物。

[0156] 在一些情况下,本公开提供在单一样品体积中能够明确地检测处于存在或不存在

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的任意组合的1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或1000个分析物中的每个分析物的存在或不存在的测定而无需固定化、分离、质谱法、或解链曲线分析。在一些情况下，本公开提供在单一样品体积中能够明确地检测处于存在或不存在的任意组合的至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或1000个分析物中的每个分析物的存在或不存在的测定而无需固定化、分离、质谱法、或解链曲线分析。在一些情况下，本公开提供在单一样品体积中能够明确地检测处于存在或不存在的任意组合的少于1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或1000个分析物的存在或不存在的测定而无需固定化、分离、质谱法、或解链曲线分析。

[0157] IV.信号

[0158] 本公开中提出的方法可以任何可定量的信号进行使用。在一些情况下，本公开提供了使用信号的单一分量(例如，强度)，以非简并性编码无限数目的目标的编码方案和方法。例如，如在上文和在实施例3中描述的，编码方案可依赖于信号强度的多路性不考虑颜色。虽然荧光探针已被用于说明此原理，但是如在本公开中其他地方描述的，所述编码方案同样适合任何其它方法，所述其它方法提供了可定量的信号，包括电化学信号和化学发光信号。

[0159] 本公开提出的方法还可以利用在至少二个维度中的信号测量结果，也被称为信号的至少两个分量的测量结果。例如，相比于上文段落中描述的、依赖于信号强度以区分分析物的编码方案，利用信号的至少两个分量(例如，颜色和强度)允许每个信号强度带宽的单位产生更多不同的代码。当利用信号的至少两个分量时，可获得单一样品体积的至少两个分量的累积测量结果。例如，在表5描述的编码方案中，每个分析物的存在导致在4个颜色(信号的第二分量)的每个颜色上的特定的强度(信号的一个分量)。这些构成信号的组合导致了可以通过测量在每个波长或波长范围内的强度来测量累积信号。然后可使用相应的编码方案或解码矩阵，将累积测量结果转换为确定分析物存在或不存在。

[0160] 在一些情况下，可定量的信号包括具有频率(波长)和振幅(强度)两者的波形。信号可以是电磁信号。电磁信号可以是声音、无线电信号、微波信号、红外信号、可见光信号、紫外光信号、x射线信号、或γ射线信号。在一些情况下，电磁信号可以是荧光信号，例如可以依据波长和强度表征的荧光发射光谱。

[0161] 在本公开的某些部分，信号依据荧光信号被描述和示例。这不意味着是限制性的，且本领域的一个普通技术人员将容易地认识到适用于荧光信号的测量的原理也适用于其它信号。例如，类似荧光信号，上文描述的任何电磁信号都可以依据波长和强度表征。荧光信号的波长也可以依据颜色描述。颜色可以通过基于在特定波长或波长范围测量强度确

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定，例如，通过确定在不同波长荧光的强度分布，和/或通过利用带通滤波器以确定特定波长范围内的荧光强度来进行确定。这样的带通滤波器通常用于各种实验室仪器，包括定量PCR器。强度可以以光电检测器测量。许多波长可被称为“信道”。

[0162] 在一些情况下，本公开中提供的方法可以用于任何信号，其中累积信号以相同的颜色、频率、吸收带、等等的构成信号衡量。然而，累积信号不需要是数字信号或随构成信号的数目和强度线性调节(scale)。例如，如果测量的物理原理是吸收，那么由于Beer-Lambert定律的指数性质，累积衰减是构成衰减的乘积而构成浓度是加性的。然后累积衰减的对数将随在每个吸收带(如果使用荧光检测，相当于颜色)上的构成浓度线性调节。因此，本发明的方法是可以应用的。本发明的方法也可用于化学发光信号和电化学信号。

[0163] 信号的数目，及测量的维数或分量的数目，也可以扩大到超出本发明的示例性的实施方案中所示的数目，导致多路能力的扩大。本公开中提供的示例性实施方案利用了利用具有测量的一个或两个分量：波长和/或强度的荧光信号所构造的编码方案。可被编码的分析物的数目可以通过增加波长和/或强度的数目而增加。可被编码的分析物的数量，也可以通过增加信号的数目而增加，例如通过组合荧光信号与电化学信号或FRET信号(荧光共振能量转移)而增加。

[0164] 在一些情况下，信号的两个以上的分量可被测量。例如，信号的3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、18、20个或更多个分量可被测量。信号的至少2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、18、或20个分量可被测量。信号的至少2个，但少于3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、18、或20个分量可被测量。在一些情况下，信号的2-3、2-4、2-5、2-6、3-5、3-6、3-8、或5-10个分量可被测量。这些额外的分量可包括动力分量，例如信号衰减的速率和光漂白速率。

[0165] 如果采用荧光信号，可被编码的分析物的数目，可以进一步通过利用额外的荧光团扩大。例如，可以使用1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个荧光团。在一些情况下，可以使用至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个荧光团。在一些情况下，可以使用少于2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个荧光团。在一些情况下，可以使用4-6、4-7、4-8、4-9、4-10、5-6、5-7、5-8、5-9、5-10、10-15、或10-20的荧光团。

[0166] 通常，可被编码的分析物的数目，可以进一步通过利用来自信号的第一分量的值或值的范围的额外的第一值(例如，强度)扩大。例如，可以使用1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个来自信号的第一分量的值或值的范围的第一值。在一些情况下，可以使用至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个来自信号的第一分量的值或值的范围的第一值。在一些情况下，可以使用少于1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、

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20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个来自信号的第一分量的值或值的范围的第一值。在一些情况下，可以使用4-20、4-6、4-7、4-8、4-9、4-10、5-6、5-7、5-8、5-9、5-10、10-15、或10-20的来自信号的第一分量的值或值的范围的第一值。

[0167] 在实例中，其中强度是被定量的信号的分量，例如当利用荧光信号时，分析物的存在可以使用多个强度或强度范围编码。例如，编码方案可以利用1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个强度或强度范围。编码方案可以利用至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、20、21、22、23、24、25、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、或更多个强度或强度范围。编码方案可以利用少于2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个强度或强度范围。在一些情况下，编码方案可利用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或5-10个强度或强度范围。

[0168] 可被编码的分析物的数目，可以进一步通过利用来自信号的第二分量的值或值的范围的额外的第二值(例如，波长)扩大。例如，可以使用1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个来自信号的第二分量的值或值的范围的第二值。在一些情况下，可以使用至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个来自信号的第二分量的值或值的范围的第二值。在一些情况下，可以使用少于1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个来自信号的第二分量的值或值的范围的第二值。在一些情况下，可以使用4-20、4-6、4-7、4-8、4-9、4-10、5-6、5-7、5-8、5-9、5-10、10-15、或10-20个来自信号的第二分量的值或值的范围的第二值。

[0169] 在其中波长是被定量的信号的分量的实例中，例如当利用荧光信号时，分析物的存在可以使用多种波长或波长范围编码。例如，可以使用1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个波长或波长范围。在一些情况下，可以使用至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个波长或波长范围。在一些情况下，可以使用少于1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个波长或波长范围。在一些情况下，可以使用4-20、4-6、4-7、4-8、4-9、4-10、5-6、5-7、5-8、5-9、5-10、10-15、或10-20的波长或波长范围。

[0170] 在一些情况下，当简并性被消除，本发明的方法能够检测在单一体积中的至少6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、

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33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、50、或更多个分析物的存在或不存在，当用于在体积中产生信号的每个试剂仅产生一个第二值(例如，每个试剂，仅发射一个波长的光)时。

[0171] 在其它情况下，当简并性被消除，本发明的方法能够检测在单一体积中的至少7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个分析物的存在或不存在，总共在该体积中使用了四个第二值(例如，共四个波长或波长的范围，其可能通过使用四个光谱可分辨的荧光团实施)。

[0172] 如在整个说明书中描述的，本文提供的测定利用对样品的累积测量结果。累积测量结果可以是，例如，强度值的单一测量结果，或在一个或更多个波长或波长的范围的强度值的测量结果。可以获得多个累积测量结果。例如，强度可以在1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个波长或波长范围测量。强度可以在至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个波长或波长范围测量。强度可以在少于1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个波长或波长范围测量。

[0173] 更普遍地，可获得关于信号的任何可定量的分量，以及关于在信号的另一个可定量的分量的信号的任何可定量的分量的累积测量结果。例如，信号的至少一个第一分量可以在1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个信号的第二分量测量。信号的至少一个第一分量可以在至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个信号的第二分量测量。信号的至少一个第一分量可以在少于1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个信号的第二分量测量。

[0174] 如本公开明显显示的，待检测的每个分析物可被编码为利用任意数目的信号的合适的分量，或任意数目的信号的代码。例如，待检测的每个分析物可以被编码在1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个信号的分量或信号中。在一些情况下，待检测的每个分析物可以被编码在至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个信号的分量或信号中。在一些情况下，待检测的每个分析物可以被编码在少于1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个信号的分量或信号中。在一些情况下，可以使用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、或10-20的信号的分量或信

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号编码每个分析物。在编码方案中每个分析物可以由相同数目的信号的分量，或信号，或不同数目的信号的分量，或信号编码。

[0175] A.探针、引物、荧光团、和猝灭剂

[0176] 本公开中提供的一些方法利用在分析物存在时产生信号的试剂。任何合适的试剂可用于本发明。通常，试剂将具有分析物特异性成分，及在分析物存在时产生信号的分量。在一些情况下，这些试剂被称为探针。探针可以是杂交探针。杂交探针可以是连接到荧光团和猝灭剂(例如，TAQMAN探针)的寡核苷酸探针。

[0177] 本发明的方法可使用一种或更多种试剂或探针以检测每个分析物的存在或不存在。例如，可以使用1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个探针以检测每个分析物的存在或不存在。在一些情况下，可以使用至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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个探针，以检测每个分析物的存在或不存在。在一些情况下，可以使用少于2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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个探针以检测每个分析物的存在或不存在。在一些情况下，用于检测每个分析物的存在或不存在的探针的数目是1-10、2-9、2-8、2-7、2-6、2-5、2-4、1-3、或1-4。

[0178] 在一些情况下，样品与4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个探针接触，以检测所有分析物的存在或不存在。在一些情况下，样品与至少4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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或更多个探针接触，以检测所有分析物的存在或不存在。在一些情况下，样品与少于4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个探针接触，以检测所有分析物的存在或不存在。在一些情况下，样品所接触的用以检测所有分析物的存在或不存在的探针的数目是4-50、4-40、4-30、4-20、5-15、5-10、或3-10。

[0179] 如上文描述的，连接到荧光团和猝灭剂的寡核苷酸探针可用于检测多核苷酸扩增测定中分析物的存在。只要猝灭剂和荧光团接近，猝灭剂就猝灭由光源激发荧光团所发射的荧光。寡核苷酸探针的序列被设计为互补于分析物中存在的多核苷酸序列，并因此，能够与分析物中存在的多核苷酸序列杂交。在包括引物的核酸扩增反应(例如，聚合酶链式反应)中可执行寡核苷酸探针的杂交。通过DNA聚合酶延伸引物后，聚合酶的5'至3'外切酶活性降解探针，释放荧光团和猝灭剂至介质中。荧光团和猝灭剂之间的接近被破坏，且来自荧光团的信号不再猝灭。因此，检测到的荧光的量是分析物存在的量的函数。如果没有分析物存在，探针将不与分析物杂交，且荧光团和猝灭剂将保持极为接近。很少信号或没有信号会产生。

[0180] 寡核苷酸探针的每个探针可具有一个或多个荧光团和猝灭剂。例如，在一些实施方案中，寡核苷酸探针可包括1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、

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21、22、23、24、25、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、或更多个荧光团。寡核苷酸探针可包括至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个荧光团。寡核苷酸探针可包括少于2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个荧光团。

[0181] 寡核苷酸探针可包括1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个猝灭剂。寡核苷酸探针可包括至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个猝灭剂。寡核苷酸探针可包括少于2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个猝灭剂。

[0182] 探针和猝灭剂至探针的连接可以在相同的反应，或在系列的反应中执行。可以执行一系列的反应以使用至少一种荧光团标记探针，且反应产物可被混合以产生具有不同荧光团的探针的混合物。

[0183] 如果寡核苷酸探针包括两个或更多个荧光团，这些荧光团可以被布置为允许Förster(荧光)共振能量转移(FRET)在荧光团之间发生。简单地说，FRET是在荧光团之间的能量转移的机制。使用基于FRET的探针允许一个激发源通过单一的荧光团的激发，产生两个不同的荧光发射信号。例如，本文描述的方法可使用配对的探针，其中在配对中的一个探针连接到荧光团和猝灭剂，且第二探针连接到两个荧光团(足够接近用于FRET发生)和猝灭剂。可以使用提供用于FRET的荧光团和猝灭剂任意组合。这种方法使得可以与激发源使用的荧光探针的数目加倍，这是因为单一的激发源可用于产生两个信号：一个来自具有一个荧光团的探针，且一个来自具有足够接近用于FRET发生的两个荧光团的探针。例如，使用表8中描述的编码方法，通过将表8中的每个探针与对应的FRET探针配对，明确地可检测的分析物的数目可从16增加到32。

[0184] 引物可以是对特定分析物特异性的，且能够扩增与探针互补的区域。在一些情况下，使用的引物的配对的数目等于探针的数目。在其它情况下，使用的探针的数目可超过使用的引物配对的数目。在又另一些情况下，使用的引物配对的数目可超过使用的探针的数目。在一些情况下，待分析的样品可与1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个引物的配对接触。在一些情况下，待分析的样品可与至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个引物的配对接触。在一些情况下，待分析的样品可与少于2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个引物的配对接触。在一些情况下，引物的配对的数目是2-10、3-15、4-20、3-10、4-10、5-10、6-8、或6-10。

[0185] 引物可扩增不同数目的杂交探针在其中杂交的多核苷酸的区域。例如，引物的至

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少一个配对可以扩增与至少2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个杂交探针互补的区域。在一些情况下，引物的所有所述配对可以扩增与至少2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个杂交探针互补的区域。

[0186] 在一个实例中，第一探针可以以荧光团5'-荧光素酰胺(5'-FAM)和猝灭剂黑洞猝灭剂1(BHQ-1)标记。第二探针可以以极为接近(例如，连接到)青色素5.5(Cy5.5)和猝灭剂黑洞猝灭剂3(BHQ-3)的5'-FAM标记。根据经由聚合酶的核酸酶活性探针的消化，从单一激发波长(例如，470nm)产生两个荧光信号。来自第一探针的荧光团在大约520nm发出荧光。在第二探针上的荧光团经受FRET。供体荧光团FAM通过激发光源(例如，470nm)激发，且转移其能量到受体荧光团Cy5.5。受体荧光团在约705nm发射。这些荧光团和猝灭剂仅是示范性的。能够经受FRET的任何荧光团，及可以猝灭荧光的任何猝灭剂适合于本发明使用。生产基于FRET探针的方法，在2009年出版的Jothikumar等人的BioTechniques，第46(7):519-524页中进行了描述。

[0187] 在一些实施例中，单一的杂交探针可用于每个分析物。为了利用多颜色编码(例如，如表3中所示)，每个探针可以以多个荧光团在预确定的比例标记。比例可被确定，在相同的颜色中，使得阳性对照信号与相同的强度的其它阳性对照信号提供相同的强度。这种方法减少了必须被合成的探针的数目，且可能比利用多个探针的方法更便宜地配置。此外，在杂交探针的情况下，相比多个探针，一个杂交探针配合分析物的序列更容易。

[0188] 虽然本发明的许多方面使用基于核酸的探针被例举，但是本领域的一个普通技术人员将容易地认识到，其它形式的探针将与在本公开中描述的本发明同样一起工作得很好。例如，对分析物特异的结合分子可用作探针。非限制性的示例性的结合分子包括识别的分析物，并在分析物存在时产生信号的抗体。

[0189] 在利用荧光标记的本发明的实施方案中，可以使用任何合适的荧光标记。适用于本发明使用的示例性的荧光标记包括罗丹明、对甲氨基酚、荧光素、硫代荧光素(thiofluorescein)、氨基荧光素、羧基荧光素、氯代荧光素、甲基荧光素、磺基荧光素、氨基对甲氨基酚(aminorhodol)、羧基对甲氨基酚(carboxyrhodol)、氯代对甲氨基酚(chlororhodol)、甲基对甲氨基酚(methylrhodol)、磺基对甲氨基酚(sulforhodol)、氨基罗丹明(aminorhodamine)、羧基罗丹明、氯代罗丹明(chlororhodamine)、甲基罗丹明(methylrhodamine)、磺基罗丹明(sulforhodamine)、及硫代罗丹明(thiorhodamine)、青色素(cyanine)、吲哚碳青色素、氧碳青色素(oxacarbocyanine)、噻碳青色素(thiacarbocyanine)、部青色素(merocyanine)、青色素2、青色素3、青色素3.5、青色素5、青色素5.5、青色素7、恶二唑衍生物(oxadiazole derivatives)、吡啶恶唑(pyridyloxazole)、硝基苯并恶二唑(nitrobenzoxadiazole)、苯并恶二唑(benzoxadiazole)、芘衍生物(pyrene derivatives)、级联蓝、恶嗪衍生物、尼罗红、尼罗蓝、甲酚紫、恶嗪170、吖啶衍生物、二氨基吖啶(proflavin)、吖啶橙、吖啶黄、金胺(arylmethine)衍生物、金胺、结晶紫、孔雀石绿、四吡咯衍生物、卟吩、酞青色素和胆红素。示例性的猝灭剂包括黑洞猝灭剂，如BHQ-0、BHQ-1、BHQ-2、BHQ-3；ATTO猝灭剂，如ATTO

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540Q、ATTO 580Q和ATTO 612Q;等等。

[0190] 可以与本发明使用的荧光团不限于本文描述的任何荧光团。例如,具有改进性能的荧光团不断发展,且这些荧光团可以很容易地与本公开提供的方法使用。这种改进的荧光团包括量子点,在单一波长被激发后,其可以以不同的波长发射能量。使用这样的荧光团的优点在于,仅需要一个单一的激发源,但是许多不同的信号可以被定量,例如,根据颜色和强度被定量。此外,具有窄的发射光谱的荧光团将对本文描述的方法特别有用,因为这样的荧光团可被包括在其发光光谱之间很少重叠或没有重叠的多路测定中,从而提供更多的"颜色"并显著地提高编码的分析物的总体数目。

[0191] 在一些情况下,一种或更多种试剂被冻干。任何合适的试剂可以被冻干。例如,探针、引物、酶、抗体或用于检测的任何其它试剂可被冻干。在一些情况下,包括分析物的样品也可被冻干。冻干可能是有用的,例如,当在发展中地区分配试剂和/或样品时,在这些地区获得冷库是昂贵的、不易利用的、或不能可靠地利用的。在一个实例中,冻干的试剂包括在本公开中描述的或以其它方式适合于本发明使用的任何探针或分析物特异性试剂。在另一个实例中,冻干的试剂包括PCR引物。在又另一实例中,冻干的试剂包括适合用于执行PCR反应的试剂。

[0192] V.分析技术与仪器

[0193] 本文提供的方法适合与各种检测方法使用。例如,可应用的方法使用测量荧光信号的波长和强度的分析技术。这可以通过测量整个光谱波长的信号的强度实现,或通过使用限制某些波长的光的带通滤波器,从而仅允许某些波长的光到达光电检测器实现。许多实时PCR和定量PCR仪器包括激发光源和带通滤波器,其能够检测在四种颜色(例如,蓝色、绿色、黄色和红色)上的荧光信号。因此,本发明的方法可以使用本领域广泛使用的仪器容易地应用。重要的是,本文提供的方法和组合物可用于通过获得单一溶液的累积测量结果来检测多种分析物。没有分离的必要。本发明不需要使用珠或固相。当然,本领域的一个普通技术人员应当理解,如果需要的话,本发明可以结合分离、珠、或固相使用。

[0194] 图5显示了可用于获得对本发明的方法有用的累积测量结果的仪器的配置的两个实例。参考图5,带有配置为检测波长和强度两者的检测器(例如,荧光计)的仪器示于501。参考501,至少一个激发光源502被引导到含有分析物和在分析物504存在时产生信号的试剂的室503中。如在其他地方描述的,分析物可以是核酸,且产生信号的试剂可以是包括荧光团和猝灭剂的杂交探针。如果分析物存在,则产生发射信号505。在501中所示的配置中,该发射信号的波长和强度通过能够产生荧光发射光谱506的检测器在整个光谱上测量。

[0195] 波长和强度也可以使用光电检测器和带通滤波器的组合确定。该配置在本领域中已知的几种热循环仪中使用。参考图5,具有带通滤波器和光点检测器的仪器在507中描绘。参考507,至少一个激发光源508被引导到含有分析物和在分析物510存在时产生信号的试剂的室509中。如在其他地方描述的,分析物可以是核酸,且产生信号的试剂可以是包括荧光团和猝灭剂的杂交探针。如果分析物存在,则产生发射信号511。在507所示的配置中,带通滤波器512、513和514被用于限制光的通过,以在特定的波长范围内发光。例如带通滤波器512可以限制光的通过,以在波长范围1内发光。带通滤波器513可以限制光的通过,以在波长范围2内发光。带通滤波器514可以限制光的通过,以在波长范围3内发光。任何数目的带通滤波器(例如,1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、

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24、25、26、27、28、29、30、或更多)可用于本发明。此外,在507中带通滤波器的位置不意味着是限制性的。在一些情况下,发射的光可在任何时间通过多于一个的带通滤波器。在其它情况下,带通滤波器可以被配置为使得所有发射的光在一个时间仅通过一个带通滤波器。通过带通滤波器后,过滤的光515、516和517通过光电检测器检测。例如,过滤的光515可能是在波长范围1内的光,过滤的光516可能是在波长范围2内的光,且过滤的光517可能是在波长范围3内的光。然后每个过滤的光515、516、及517的强度可以通过光电检测器518定量。

[0196] 本公开中描述的方法可与各种扩增方法兼容,包括定量PCR(qPCR)法、终点PCR法、逆转录酶PCR、及数字PCR法。数字PCR法(例如,微滴数字(BIORAD)和动态阵列(FLUIDIGM))产生高度敏感的多核苷酸拷贝数目的定量。本文提供的方法可以容易地集成到这些系统中,通过允许在微滴或动态阵列中的多路显著地扩大其工作能力。

[0197] 类似地,本公开中描述的方法可在实时PCR测定中应用。例如,随着PCR运行完成,实时数据可以被完全记录。然后端点值可被解码以指示分析物的存在或不存在。通过检测该颜色的实时曲线的二阶导数的最大值,可对每个颜色分析各个循环阈值(Ct)的值。因为特定的分析物被扩增,荧光团以与该序列编码的颜色多样性相同的比例从其相应的探针释放。因此,如1100代码在蓝色和绿色中将具有相同的Ct,而1400代码将具有领先其蓝色Ct2个循环的绿色Ct。这种额外的数据能够测定每个分析物的标识和起始量。

[0198] 本文描述的方法也可以直接在组织样品上使用。例如,可以获得组织样品,且使用光刻法在组织样品上直接建造孔(well)。组织样品可以被事先固定以建造孔。然后可通过分配适当的试剂进入组织样品中的孔中以分析组织样品。本公开中描述的编码和解码方法可用于蚀刻到组织中的孔中的分析物的多路检测。每个孔可以对应于单一细胞或若干细胞,当对组织的不同部分执行分析物分析时,提供优异的空间分辨率。这个方法可以被用于检测相同组织的不同区域的分析物。

[0199] 在一些情况下,仪器可被修改或构建,例如,以提供在多波长的额外的激发光源,和/或提供额外的带通滤波器或确定完整的光谱的能力。包括额外的激发源将允许更大数量的各种荧光团的激发。包括额外的带通滤波器,或者修改或构建仪器能够确定整个光谱,允许检测来自荧光团的更多种发射。这些技术可用于增加可以用于本公开中描述的方法的荧光团的数目,且因此可以增加被同时检测的分析物的数目。

[0200] 在一些情况下,本公开中描述的方法利用终点PCR法。然而,在一些情况下,结果可在终点前获得。例如,当尽快需要结果时,这可能是有利的。例如,在一种执行校准实验的情况下,其中分析分析物的不同的起始浓度,以确定信号达到饱和其循环数,其为分析物的起始量的函数。使用这种数据,实时监控PCR反应的计算机,可被编程以根据特定系统的检测极限(LOD)的限制搜索达到最大的循环数的饱和。如果到最大循环数时,分析物没有扩增(例如,除了可能存在的阳性对照),那么对于该分析物的结果是阴性。如果到最大循环数时,分析物有扩增,那么对于该分析物的结果是阳性,且在每个颜色中的饱和强度可以被用于使用编码方案解码结果。在这两种情况下,没有必要运行PCR反应超过由LOD为仪器设定的循环的数目。尽管PCR方法已被用于说明这种定量方法,本领域的一个普通技术人员将容易认识到,类似的原理可以在任何催化体系中应用,其中分析物的起始量限制了反应速率,且反应速率可以通过在一段时间内信号强度的演变被测量和报告。

[0201] VI.组合物和试剂盒

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[0202] 本公开还提供了与本文描述的方法使用的组合物和试剂盒。组合物可以包括本文描述的任何成分、反应混合物和/或中间体，以及任意组合。例如，本公开提供了与本文提供的方法一起使用的检测试剂。可提供任何合适的检测试剂，包括如在本说明书中其他地方描述的，以荧光团和猝灭剂和引物标记的杂交探针。

[0203] 在一些情况下，组合物包括使用四个荧光团检测至少七个分析物的试剂。在一些情况下，组合物包括使用四个荧光团检测至少5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个分析物的试剂。在一些情况下，组合物包括使用五个荧光团检测至少6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个分析物的试剂。在一些情况下，组合物包括使用六个荧光团检测至少7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个分析物的试剂。在一些情况下，组合物包括使用七个荧光团检测至少8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个分析物的试剂。

[0204] 在一些情况下，组合物包括引物。组合物可包括至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、或50个引物配对。

[0205] 本发明还提供了用于实施本发明的方法的试剂盒。因此，各种试剂盒在合适的包装中提供。试剂盒可用于本文描述的任何一个或更多个用途，且，相应地，可含有用于检测多个分析物中的每个分析物的存在或不存在的指令。试剂盒可包括编码方案、或解码矩阵，以帮助用户转换累积测量结果为表明多个分析物中的每个分析物的存在或不存在的结果。试剂盒可以是诊断试剂盒，例如，适合用于包括本文列举的分析物的任何分析物的检测的诊断试剂盒。试剂盒可以包含任何本公开中提供的组合物，包括上文列举的那些。

[0206] 图6显示了本发明的示例性试剂盒的示意图。参考图6，显示了包括盒体601的试剂盒，盒体601含有用于分析物(例如，包括荧光团和猝灭剂的多核苷酸探针)的检测的试剂602，及用于核酸的扩增的引物603。试剂盒也可以包含任何其他试剂，例如适合于执行扩增反应的试剂604。在601中描绘的实施方案中，用于分析物的检测的试剂及引物各自包含在单一体积(如管或瓶)中。然而，这些试剂也可以以单独的体积提供，如605中所示的盒体所描绘的。盒体605包含七个单独的体积。三个体积606、607和608含有用于分析物的检测的试剂(或这类试剂的混合物)，且三个体积609、610和611含有引物(或引物的混合物)。试剂盒还可以含有适合于执行扩增反应的试剂604。

[0207] 提供图6仅用于说明的目的。用于分析物(例如，探针)的检测的任何数目的试剂，及任何数目的引物可以被包括在单一的管或瓶中，使其适合应用。例如，在一些实施方案中，在单一的瓶中可以包括1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个试剂，用于分析物的检测。在其它情况下，在单一的瓶或管中可以包括1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个引

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物，用于扩增。在一些情况下，引物和探针可在相同的瓶或管中提供。例如，每对引物配对可以提供1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个探针。在其它情况下，每对探针配对可以提供1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个引物。试剂盒可以包含1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、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、或更多个瓶或管。对照分析物，及其相关的检测试剂和引物，也可以包括在试剂盒中。这些可以包括在单独的瓶或管中，或者例如，包括在提供用于扩增反应的试剂的瓶或管604中。如在本说明书中其他地方描述的，可以冻干试剂盒中的任何试剂。

[0208] VII.系统和软件

[0209] 本公开的方法还可以作为系统的一部分执行。例如，可以通过包含在计算机可读介质中的软件执行本文描述的方法的一个或更多个步骤。软件可用于编码分析物、检查简并性、减少或消除简并性、和/或产生解码矩阵。软件也可以用于将仪器(例如，PCR器)上作出的累积测量结果转换为关于分析物的存在或不存在的信息。例如，参考图4，计算机401可以连接到输入装置403、显示器402和打印机404。计算机可以执行本公开中描述的方法的任意一个方法的一个或更多个步骤，例如包括，编码方法410、分析方法420和解码方法430。计算机可减少或消除简并性，例如，作为编码方法410的一部分。类似地，计算机可以产生通过设计是非简并的编码方案，例如使用数学迭代设计的编码方案。计算机也可以连接到网络405。网络可以是局域网和/或因特网。网络可以用于在不同位置的多个计算机上执行本发明的方法。例如，一台计算机可以执行编码，另一台计算机可执行测量，且又一台计算机可执行解码。一台计算机可执行这些方法的任何方法或全部。网络可用于在全世界传输使用本公开中描述的方法所获得的信息，例如跨越国界。

[0210] 在一个实例中，软件被嵌入在执行测量的仪器中，例如热循环仪。在另一个实例中，软件被安装在连接到执行测量的仪器的计算机上，例如连接到热循环仪的计算机。在另一个实例中，软件是“云”，即在另一台计算机可通过网络与其通信的计算机上。

[0211] 上文描述的每个元件可被连接到控制器，所述控制器与每个元件通信，并协调它们的操作。例如，控制器可启动将一系列的分析物编码为颜色和强度的值。如本公开全文所描述的，控制器可以执行软件，以通过从编码矩阵中除去一个或更多个潜在的分析物代码来减少或消除简并性。然后，控制器可以自动地从探针的制造商订购对应该代码的探针。可替代地，控制器可以操作可以合成相应的探针的寡核苷酸合成仪。分析了在仪器上的样品(其也可以是自动的)之后，控制器可以使用解码矩阵处理分析的结果。然后，控制器可将这些结果提供给用户。

[0212] VIII.服务

[0213] 本文提供的方法也可以作为服务执行。例如，服务提供者可以获得客户希望分析的多个分析物的标识。然后服务提供者可以通过本文所述的任何方法编码每个待检测的分析物，并向客户提供适当的试剂用于测定。客户也可以执行测定，并向服务提供者提供结果以解码。然后服务提供者可以向客户提供解码的结果。客户还可以通过与安装在本地(在客

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户所在地)或远程(例如,在通过网络可达的一台服务器上)的软件交互,;来编码分析物、产生探针、和/或解码结果。示例性的客户包括临床实验室、医生、食品和消费品制造商、工业制造商(例如,石油公司)等等。客户或团体可以是有需要或希望使用本发明的方法、系统、组合物和试剂盒的任何合适的客户或团体。

[0214] 实施例

[0215] 实施例1:一般材料和方法

[0216] 1.一般试剂

[0217] TE缓冲液,pH7(LIFE TECHNOLOGIES,Carlsbad,CA);无RNA酶超纯水(LIFE TECHNOLOGIES,Carlsbad,CA);Taq 5x预混液(FISHER SCIENTIFIC COMPANY,Tustin,CA)。

[0218] 2.DNA序列、引物和探针

[0219] 选择了来自临床相关的生物体的五种核酸用于示例性的研究:(1)人类免疫缺陷病毒1(HIV-1);(2)恶性疟原虫(疟疾);(3)单纯性疱疹病毒2(HSV-2);(4)结核分枝杆菌(TB);和(5)登革热病毒3型(登革热)。

[0220] 来自HIV-1的基因组的诊断相关的两个区域,p17和聚蛋白酶,分别选自Los Alamos国家实验室的HIV-1参考序列。来自恶性疟原虫ChR 7基因的诊断序列从UCSC恶性疟原虫基因组浏览器获得。单纯性疱疹病毒2序列从欧洲分子生物学库(European Molecular Biology Library)中获得的序列合成。相似地,结核分歧杆菌中的rpoB基因的诊断序列合成自从欧洲分子生物学库获得的序列。登革热病毒3型的PCR诊断序列获得自美国国家卫生研究院(National Institute of Health)的健康基因序列数据库。

[0221] 由INTEGRATED DNA TECHNOLOGIES(IDT)合成寡核苷酸。使用来自IDT的OLIGO分析仪选择用于每个分析物的探针和引物配对。对所有分析物合成包含在5'端的荧光团和在3'端的猝灭剂的感测探针。冻干所有的寡核苷酸,并在使用前在TE缓冲液中重建。表9-14显示了六个分析物的每个分析物的序列信息、目标序列、引物、和探针。

[0222] 表9.HIV-1聚蛋白酶的序列信息。

[0223]

序列信息	从碱基 2253-2550 合成的 HIV-1 多蛋白酶 198mer
来源	HIV-1 参考序列, Los Alamos 国家实验室
目标序列 (3'至 5')	GGAAGCTCTATTAGATACAGGAGCAGATGAT ACAGTATTAGAAGAAATGA GTTTGCCAGGAAGATGGAAACCAAAAATGAT AGGGGGAATTGGAGGTTTT ATCAAAGTAAGACAGTATGATCAGATACTCA TAGAAATCTGTGGACATAA AGCTATAGGTACAGTATTAGTAGGACCTACA CCTGTCAACATAATTGG
正向引物 (3'至 5')	GGAAGCTCTATTAGATACAGGAGCAG
反向引物 (3'至 5')	CCAATTATGTTGACAGGTGTAGGTCC
探针 1 (3'至 5')	/56-FAM/TGAGTTTGCCAGGAAGATGGAAAC CA/3BHQ_1/

[0224] 表10.HIV p17的序列信息。

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[0225]

序列信息	从碱基 790-1186 合成的 HIV-1 P17 199mer
来源	HIV-1 参考序列、Los Alamos 国家实验室
目标序列（3’至5’）	CAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATA TAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAG ACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAG AAAAAAGCACAGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGTCA

[0226]

正向引物（3’至5’）	CAGCTACAACCATCCCTTCAGACA
反向引物（3’至5’）	TGACCTGATTGCTGTGTCCTGTGT
探针 1（3’至 5’）	/**56-FAM**/AGCAACCCTCTATTGTGTGCATCAAAGG/3BHQ_1
探针 2（3’至 5’）	/**5Cy3**/AAAGCACAGCAAGCAGCAGCTGA/3BHQ_2/

[0227] 表11.疟原虫ChR 7的序列信息。

[0228]

序列信息	从碱基 1139138-1141223 合成的疟原虫 ChR7 199mer
来源	UCSC 恶性疟原虫基因组浏览器网关
目标序列（3’至5’）	GCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAGAGGGAGCCTGAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTAAATTACCCAATTCTAAAGAAGAGAGGTAGTGACAAGAAATAACAATGCAAGGCCAATTTAAAACCTTCCCAGAGTAACAATTGGAGGGCAAGTCTGGTG
正向引物（3’至5’）	GCCTAACATGGCTATGACGGGTAA
反向引物（3’至5’）	CACCAGACTTGCCCTCCAATTGTT
探针 1（3’至 5’）	/**56-FAM**/ATTCCGGAGAGGGAGCCTGAGAAATA/3BHQ_1/
探针 2（3’至 5’）	/**56-ROXN**/AAGGAAGGCAGCAGGCGCGTAAATTA/3BHQ_2/

[0229] 表12.单纯性疱疹病毒2的序列信息。

[0230]

序列信息	从 HSV-2 基因组合成的 HSV-2 193mer
来源	EMBL 库 AJ303204
目标序列（3’至5’）	TCAGCCCATCCTCCTTCGGCAGTATGGAGGGTGTCGCGGCGGCGAGCCGCCGTCCCCAAAGACGTGCGGGTCGTACACGTACACGTACCAGGGCGGCGGGCCTCCGACCCGGTACGCTCTCGTAAATGCTTCCCTGCTGGTGCCGATCTGGGACCGCGCCGCGGAGACATTCGAGTACCAGATCGAACTCGG
正向引物（3’至5’）	TCAGCCCATCCTCCTTCGGCAGTAT
反向引物（3’至5’）	CCGAGTTCGATCTGGTACTCGAATGT
探针 1（3’至 5’）	/**56-FAM**/AAAGACGTGCGGGTCGTACACGTACA/3BHQ_1/
探针 2（3’至 5’）	/**5Cy5**/TAAATGCTTCCCTGCTGGTGCCGAT/3IAbRQSp/

[0231] 表13.结核分枝杆菌rpoB的序列信息。

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[0232]

序列信息	从结核分枝杆菌 rpoB 基因组合成的结核分枝杆菌 rpoB 200mer
来源	EMBL 库 GQ395623

[0233]

目标序列（3’至5’）	GAGTGCAAAGACAAGGACATGACGTACGCGGCCCCGCTGTT CGTCACGGCCGAGTTCATCAACAACAACACCGGCGAGATCA AGAGCCAGACGGTGTTCATGGGTGACTTCCCGATGATGACCG AGAAGGGCACCTTCATCATCAACGGCACCGAGCGCGTCGTG GTCAGCCAGCTGGTCCGCTCGCCCGGTGTGTACTT
正向引物（3’至5’）	GAGTGCAAAGACAAGGACATGACG
反向引物（3’至5’）	AAGTACACACCGGGCGAGC
探针1（3’至5’）	**/56-FAM**/CGCTGTTCGTCACGGCCGAGTTCAT/3BHQ_1/
探针2（3’至5’）	**/5Cy3**/AGATCAAGAGCCAGACGGTGTTCATG/3BHQ_2/
探针3（3’至5’）	**/5Cy5**/AAGGGCACCTTCATCATCAACGGCA/3IAbRQSp/

[0234] 表14.登革热病毒3型的序列信息。

[0235]

序列信息	从登革热病毒 3 型基因组合成的登革热 3 型 200mer
来源	GenBank M93130
目标序列（3’至5’）	ATGCCAACTGTGATTGAGCACTTAGAAAGACTACAAAGGAA ACATGGAGGAATGCTTGTGAGAAATCCACTCTCACGAAACTC CACGCACGAAATGTATTGGATATCCAATGGTACAGGCAACATC GTCTCATTCACAATGACACACAGGAGACCCACCATAGAGAAA GATGTGGATTTAGGAGCAGGAACCCGACATGT
正向引物（3’至5’）	ATGCCAACTGTGATTGAGCACT
反向引物（3’至5’）	ACATGTCGGGTTCCTGCTCCTAAA
探针1（3’至5’）	**/56-FAM**/ACAAAGGAAACATGGAGGAATGCTTGTGA/3BHQ_1/
探针2（3’至5’）	**/5Cy3**/ACTCTCACGAAACTCCACGCACGAAA/3BHQ_2/
探针3（3’至5’）	**/56-ROXN**/ACAATGACACACAGGAGACCCACCAT/3BHQ_2/
探针4（3’至5’）	**/5Cy5**/GGATATCCAATGGTACAGGCAACATCGT/3IAbRQSp/

[0236] 3.聚合酶链式反应

[0237] 所有的PCR反应在ROCHE 480LIGHTCYCLER仪器上执行。以在95℃,60秒的热启动，执行45个循环。变性、退火(annealing)和延伸的循环条件为分别在95℃、65℃和70℃分别为45秒、50秒和60秒。每个实验以15μL的反应体积一式五份运行。每个退火步骤后，在以下通道中获得荧光测量结果：483nm至533nm(FAM)、523nm至568nm(Cy3)、558nm至610nm(ROX)、及615nm至670nm(Cy5)。在热启动和45个热循环结束后也可以获得荧光测量结果。这两个测量结果之间的荧光强度的变化(热启动后，及在45个循环结束时)确定终点荧光信号。

[0238] 实施例2：基于每个分析物使用多于一个颜色的编码方法同时检测来自病原体的多核苷酸标记

[0239] 本实施例描述了选自HIV(2个多核苷酸)、HSV-2、分枝杆菌、疟原虫、和登革病毒的最多四种多核苷酸的同时检测。使用表15中描述的试剂，装配(assemble)了14个PCR反应。

[0240] 表15. 14个PCR反应的试剂。

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[0241]

反应数	试剂类型	试剂	浓度	体积
1		超纯水	-	34 μL
		Taq 5x 预混液	-	42 μL
	模板	聚蛋白酶模板	17 nM	2 μL
	引物	聚蛋白酶 FWD 引物	1 nM	2 μL
		蛋白酶 RVS 引物	1 nM	2 μL
	探针	聚蛋白酶探针 1	1 nM	2 μL
2		超纯水	-	28 μL
		Taq 5x 预混液	-	42 μL
	模板	疟疾模板	15 nM	2 μL
		疱疹模板	17 nM	2 μL
		TB 模板	16 nM	2 μL
		登革热病毒模板	17 nM	2 μL
	引物	聚蛋白酶 FWD 引物	1 nM	2 μL
		蛋白酶 RVS 引物	1 nM	2 μL
	探针	聚蛋白酶探针 1	1 nM	2 μL
3		超纯水	-	32 μL
		Taq 5x 预混液	-	42 μL
	模板	疟疾模板	15 nM	2 μL
	引物	疟疾 FWD 引物	1 μM	2 μL
		疟疾 RVS 引物	1 μM	2 μL
	探针	疟疾探针 1	1 μM	2 μL
		疟疾探针 2	1 μM	2 μL
4		超纯水	-	26 μL
		Taq 5x 预混液	-	42 μL
	模板	聚蛋白酶模板	17 nM	2 μL
		疱疹模板	17 nM	2 μL
		TB 模板	16 nM	2 μL
		登革热病毒模板	17 nM	2 μL
	引物	疟疾 FWD 引物	1 μM	2 μL

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[0242]

		痘疾 RVS 引物	1 μM	2 μL
	探针	痘疾探针 1	1 μM	2 μL
		痘疾探针 2	1 μM	2 μL
5		超纯水	-	32 μL
		Taq 5x 预混液	-	42 μL
	模板	疱疹模板	17 nM	2 μL
	引物	疱疹 FWD 引物	1 μM	2 μL
		疱疹 RVS 引物	1 μM	2 μL
	探针	疱疹探针 1	1 μM	2 μL
		疱疹探针 2	1 μM	2 μL
6		超纯水	-	26 μL
		Taq 5x 预混液	-	42 μL
	模板	聚蛋白酶模板	17 nM	2 μL
		痘疾模板	15 nM	2 μL
		TB 模板	16 nM	2 μL
		登革热病毒模板	17 nM	2 μL
	引物	疱疹 FWD 引物	1 μM	2 μL
		疱疹 RVS 引物	1 μM	2 μL
	探针	疱疹探针 1	1 μM	2 μL
		疱疹探针 2	1 μM	2 μL
7		超纯水	-	30 μL
		Taq 5x 预混液	-	42 μL
	模板	TB 模板	16 nM	2 μL
	引物	TB FWD 引物	1 μM	2 μL
		TB RVS 引物	1 μM	2 μL
	探针	TB 探针 1	1 μM	2 μL
		TB 探针 2	1 μM	2 μL
		TB 探针 3	1 μM	2 μL
8		超纯水	-	30 μL
		Taq 5x 预混液	-	42 μL
	模板	聚蛋白酶模板	17 nM	2 μL
		痘疾模板	15 nM	2 μL
		疱疹模板	17 nM	2 μL
		登革热病毒模板	17 nM	2 μL
	引物	TB FWD 引物	1 μM	2 μL
		TB RVS 引物	1 μM	2 μL
	探针	TB 探针 1	1 μM	2 μL
		TB 探针 2	1 μM	2 μL
		TB 探针 3	1 μM	2 μL
9		超纯水	-	28 μL
		Taq 5x 预混液	-	42 μL
	模板	登革热病毒模板	17 nM	2 μL
	引物	登革热病毒 FWD 引物	1 μM	2 μL

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[0243]

		登革热病毒 RVS 引物	1 μM	2 μL
	探针	登革热病毒探针 1	1 μM	2 μL
		登革热病毒探针 2	1 μM	2 μL
		登革热病毒探针 3	1 μM	2 μL
		登革热病毒探针 4	1 μM	2 μL
10		超纯水	-	22 μL
		Taq 5x 预混液	-	42 μL
	模板	聚蛋白酶模板	17 nM	2 μL
		痄疾模板	15 nM	2 μL
		疱疹模板	17 nM	2 μL
		TB 模板	16 nM	2 μL
	引物	登革热病毒 FWD 引物	1 μM	2 μL
		登革热病毒 RVS 引物	1 μM	2 μL
	探针	登革热病毒探针 1	1 μM	2 μL
		登革热病毒探针 2	1 μM	2 μL
		登革热病毒探针 3	1 μM	2 μL
		登革热病毒探针 4	1 μM	2 μL
11		Taq 5x 预混液	-	42 μL
	模板	聚蛋白酶模板	17 nM	2 μL
		痄疾模板	15 nM	2 μL
		疱疹模板	17 nM	2 μL
		登革热病毒模板	17 nM	2 μL
	引物	聚蛋白酶 FWD 引物	1 μM	2 μL
		聚蛋白酶 RVS 引物	1 μM	2 μL
		痄疾 FWD 引物	1 μM	2 μL
		痄疾 RVS 引物	1 μM	2 μL
		疱疹 FWD 引物	1 μM	2 μL
		疱疹 RVS 引物	1 μM	2 μL
		登革热病毒 FWD 引物	1 μM	2 μL
		登革热病毒 RVS 引物	1 μM	2 μL
	探针	聚蛋白酶探针 1	1 μM	2 μL
		痄疾探针 1	1 μM	2 μL
		痄疾探针 2	1 μM	2 μL
		疱疹探针 1	1 μM	2 μL
		疱疹探针 2	1 μM	2 μL
		登革热病毒探针 1	1 μM	2 μL
		登革热病毒探针 2	1 μM	2 μL
		登革热病毒探针 3	1 μM	2 μL
		登革热病毒探针 4	1 μM	2 μL
12		超纯水	-	2 μL
		Taq 5x 预混液	-	42 μL
	模板	聚蛋白酶模板	17 nM	2 μL
		痄疾模板	15 nM	2 μL

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[0244]

		疱疹模板	17 nM	2 μL
		TB 模板	16 nM	2 μL
	引物	聚蛋白酶 FWD 引物	1 μM	2 μL
		聚蛋白酶 RVS 引物	1 μM	2 μL
		痄疾 FWD 引物	1 μM	2 μL
		痄疾 RVS 引物	1 μM	2 μL
		疱疹 FWD 引物	1 μM	2 μL
		疱疹 RVS 引物	1 μM	2 μL
		TB FWD 引物	1 μM	2 μL
		TB RVS 引物	1 μM	2 μL
	探针	聚蛋白酶探针 1	1 μM	2 μL
		痄疾探针 1	1 μM	2 μL
		痄疾探针 2	1 μM	2 μL
		疱疹探针 1	1 μM	2 μL
		疱疹探针 2	1 μM	2 μL
		TB 探针 1	1 μM	2 μL
		TB 探针 2	1 μM	2 μL
		TB 探针 3	1 μM	2 μL
13		超纯水	-	12 μL
		Taq 5x 预混液	-	42 μL
	模板	聚蛋白酶模板	17 nM	2 μL
		疱疹模板	17 nM	2 μL
		TB 模板	16 nM	2 μL
	引物	聚蛋白酶 FWD 引物	1 μM	2 μL
		聚蛋白酶 RVS 引物	1 μM	2 μL
		疱疹 FWD 引物	1 μM	2 μL
		疱疹 RVS 引物	1 μM	2 μL
		TB FWD 引物	1 μM	2 μL
		TB RVS 引物	1 μM	2 μL
	探针	聚蛋白酶探针 1	1 μM	2 μL
		疱疹探针 1	1 μM	2 μL
		疱疹探针 2	1 μM	2 μL
		TB 探针 1	1 μM	2 μL
		TB 探针 2	1 μM	2 μL
		TB 探针 3	1 μM	2 μL
14		超纯水	-	10 μL
		Taq 5x 预混液	-	42 μL
	模板	聚蛋白酶模板	17 nM	2 μL
		痄疾模板	15 nM	2 μL
		登革热病毒模板	17 nM	2 μL
	引物	聚蛋白酶 FWD 引物	1 μM	2 μL
		聚蛋白酶 RVS 引物	1 μM	2 μL
		痄疾 FWD 引物	1 μM	2 μL

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[0245]

		疟疾 RVS 引物	1 μM	2 μL
		登革热病毒 FWD 引物	1 μM	2 μL
		登革热病毒 RVS 引物	1 μM	2 μL
	探针	聚蛋白酶探针 1	1 μM	2 μL
		疟疾探针 1	1 μM	2 μL
		疟疾探针 2	1 μM	2 μL
		登革热病毒探针 1	1 μM	2 μL
		登革热病毒探针 2	1 μM	2 μL
		登革热病毒探针 3	1 μM	2 μL
		登革热病毒探针 4	1 μM	2 μL

[0246] 参照表15，在其它分析物不存在时，反应1、3、5、7和9(分别包含HIV聚蛋白酶、疟原虫、HSV、分枝杆菌和登革热的分析物)是为每个多核苷酸分析物单独提供基线荧光强度的阳性对照。如下文更充分地描述的，在每个颜色中荧光强度的变化被用于提供色谱(图3)中每个颜色的预期累积的强度水平。反应2、4、6、8和10是阴性对照，分别用于确定非特异性扩增的程度。在这些反应中，提供的引物对提供的分析物特不是异性的。

[0247] 实验11、12、13和14是多路检测实验。如表16中表示的执行编码。在每个颜色中，对每个测定测量的荧光强度被描绘为图3的色谱中的黑色圆圈。

[0248] 表16.病原体检测的编码方案。

[0249]

	FAM	Cy3	ROX	Cy5
HIV聚蛋白酶	1	0	0	0
HIV p17	1	1	0	0
疟原虫	1	0	1	0
HSV	1	0	0	1
分歧杆菌	1	1	0	1
登革热病毒	1	1	1	1

[0250] 为了构建色谱，在每个颜色中，阳性对照的信号(反应1、3、5、7和9)被用于装配存在的序列的每个可能的组合。累积信号被绘制在称为“色谱”(图3)的图中。对应于相同颜色中相同秩的所有可能组合的累积信号被组织到其自己的“带”内。对所有四个颜色这样做产生水平(level)和带结构，针对其可以评估每个组合的实验结果(反应11、12、13和14)。图3显示了色谱的三个重复，所述色谱使用阳性对照样品构建，并以实验结果4112、3121和3102叠加在色谱上。实验结果由黑点指示。

[0251] 在反应11-14中每个颜色的强度值以任意荧光单位(AFU)测量，绘制在色谱中，且然后基于两个标准分配至带。首先，如果实验结果在带内，那么结果被分配到多路性的带(例如，在图3的最左边的色谱中为1的Cy3值)。第二，如果实验结果在两个带之间，则使用两步分析。如果实验结果在两个合理的结果之间，那么实验结果被分配到最接近的带(例如，在图3的最右边的色谱中Cy5的值2)。如果实验结果在合理的结果和不合理的结果之间，结果被分配至带的合理的结果(例如，图3的中间的色谱中FAM的值3)。图8示意性地显示了4112的合理的结果(上图)，其指示了HIV聚蛋白酶、疟原虫、单纯性疱疹、及分枝杆菌的存在；以及4110的不合理的结果(下图)，其不能被解码并指示了测定中的故障。

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[0252] 使用这些标准，实验12的输出被指定为4112，这是正确的答案。实验14的输出可以被指定为2121或3121，但2121是不合理的，因此其结果必然是3121，这是正确的答案。实验13的输出可以被指定为3102或3101，这两者都是合理的，但在Cy5通道中相比1，结果明确地更接近2，所以正确答案3102，被指定为的结果。这些结果提供了本文提供的方法的实验性验证。

[0253] 研究结果表明组合信号比可以预期的来自阳性对照信号的简单总和略低的趋势。这可能是因为信号的组合不是完美的线性。这可能是目前不能解释的色谱构建的额外效应的结果。这些效应可能与猝灭剂的绝对浓度相关。猝灭剂的绝对浓度越高，其猝灭的反应体积的百分比越大，使得释放的相同的百分比的荧光团对累积信号的贡献没有预期的那么多。这个问题一个解决方法是使用低浓度的探针，使得信号中的百分损失是可以忽略的，而不管有多少猝灭剂被释放。

[0254] 图3显示了包含多个组合的每个秩具有相对宽的带。带的宽度最终是阳性对照样品的最高和最低累积信号之间的差异。如果在特定颜色中所有阳性对照产生完全相同的荧光信号，则每个带的宽度将是零。因为代替的那些信号有所不同，得到的带是相对宽的。然而，有一种简单的手段以收紧带。如在上述实验完成的，与加载相同浓度的所有探针不同，作为代替，每个探针的浓度可被调整，使得每个得到的阳性对照信号与相同的颜色中的其它的是相同的。这种方法会显著收紧带，使其更容易确定含有分析物的样品的信号的多路性。

[0255] 实施例3：基于每个分析物使用一个颜色和不同强度的编码方法同时检测来自病原体的多核苷酸标记

[0256] 本实施例描述了选自登革病毒、HIV p17和HIV聚蛋白酶的最多三种多核苷酸的同时检测。编码方案如表11中描绘的。如表11所示，每个分析物被编码为单一颜色中荧光强度的单一值。在这种情况下，如本公开其他地方描述的，编码方案通过设计是非简并的。这通过向每个分析物分配强度值实现，所分配的强度值等于之前的分析物的累积强度值加一。当然，如在本说明书中其他地方描述的，初始分配值可以大于1，例如，以更好地区别噪声。类似地，分配给每个分析物的值可以大于之前的分析物的累积强度值加一，例如，为了增加强度之间的间隔，并使得强度分配更简单。

[0257] 表17.检测登革热病毒、HIV聚蛋白酶和HIV p17的编码方案。

[0258]

	FAM
登革热病毒	1
HIV p17	2
HIV聚蛋白酶	4

[0259] 实施例2中使用的相同的试剂(例如，模板、引物和探针)用于此处。测量了由阳性对照终点PCR反应中的各个探针产生的荧光信号，并将其用于计算将产生1x、2x、和4x的信号强度的探针的浓度。然后设置实验用于检测分析物出现的七个非空的组合。表18-24提供了这些混合物的每个的分析物PCR反应成分。

[0260] 表18.二进制FAM实验1混合液(Cocktail)：HIV聚蛋白酶、HIV p17、及登革热病毒。

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[0261]

试剂	浓度	添加的体积
超纯水	-	50 μL
Taq 5x 预混液	-	25 μL
模板	浓度	添加的体积
聚蛋白酶模板	17 nM	4 μL
P17 模板	15 nM	4 μL
登革热病毒模板	17 nM	4 μL
引物	浓度	添加的体积
聚蛋白酶 FWD 引物	1 μM	4 μL
聚蛋白酶 RVS 引物	1 μM	4 μL
P17 FWD 引物	1 μM	4 μL
P17 RVS 引物	1 μM	4 μL
登革热病毒 FWD 引物	1 μM	4 μL
登革热病毒 RVS 引物	1 μM	4 μL
探针	浓度	添加的体积
聚蛋白酶探针 1	800 nM	4 μL
P17 探针 1	400 nM	4 μL
登革热病毒探针 1	200 nM	4 μL

[0262] 表19.二进制FAM实验2混合液:登革热病毒和HIV p17。

[0263]

试剂	浓度	添加的体积
超纯水	-	54 μL
Taq 5x 预混液	-	25 μL
模板	浓度	添加的体积
聚蛋白酶模板	17 nM	-
P17 模板	15 nM	4 μL
登革热病毒模板	17 nM	4 μL
引物	浓度	添加的体积
聚蛋白酶 FWD 引物	1 μM	4 μL
聚蛋白酶 RVS 引物	1 μM	4 μL
P17 FWD 引物	1 μM	4 μL
P17 RVS 引物	1 μM	4 μL
登革热病毒 FWD 引物	1 μM	4 μL
登革热病毒 RVS 引物	1 μM	4 μL
探针	浓度	添加的体积
聚蛋白酶探针 1	800 nM	4 μL
P17 探针 1	400 nM	4 μL
登革热病毒探针 1	200 nM	4 μL

[0264] 表20.二进制FAM实验3混合液:登革热病毒和HIV聚蛋白酶。

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[0265]

试剂	浓度	添加的体积
超纯水	-	54 μL
Taq 5x 预混液	-	25 μL
模板	浓度	添加的体积
聚蛋白酶模板	17 nM	4 μL
P17 模板	15 nM	-
登革热病毒模板	17 nM	4 μL
引物	浓度	添加的体积
聚蛋白酶 FWD 引物	1 μM	4 μL
聚蛋白酶 RVS 引物	1 μM	4 μL
P17 FWD 引物	1 μM	4 μL
P17 RVS 引物	1 μM	4 μL
登革热病毒 FWD 引物	1 μM	4 μL
登革热病毒 RVS 引物	1 μM	4 μL
探针	浓度	添加的体积
聚蛋白酶探针 1	800 nM	4 μL
P17 探针 1	400 nM	4 μL
登革热病毒探针 1	200 nM	4 μL

[0266] 表21.二进制FAM实验4混合液:HIV聚蛋白酶和HIV p17。

[0267]

试剂	浓度	添加的体积
超纯水	-	54 μL
Taq 5x 预混液	-	25 μL
模板	浓度	添加的体积
聚蛋白酶模板	17 nM	4 μL
P17 模板	15 nM	4 μL
登革热病毒模板	17 nM	-
引物	浓度	添加的体积
聚蛋白酶 FWD 引物	1 μM	4 μL
聚蛋白酶 RVS 引物	1 μM	4 μL
P17 FWD 引物	1 μM	4 μL
P17 RVS 引物	1 μM	4 μL
登革热病毒 FWD 引物	1 μM	4 μL
登革热病毒 RVS 引物	1 μM	4 μL
探针	浓度	添加的体积
聚蛋白酶探针 1	800 nM	4 μL
P17 探针 1	400 nM	4 μL

[0268]

登革热病毒探针 1	200 nM	4 μL

[0269] 表22.二进制FAM实验5混合液:登革热病毒。

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[0270]

试剂	浓度	添加的体积
超纯水	-	58 μL
Taq 5x 预混液	-	25 μL
模板	浓度	添加的体积
聚蛋白酶模板	17 nM	-
P17 模板	15 nM	-
登革热病毒模板	17 nM	4 μL
引物	浓度	添加的体积
聚蛋白酶 FWD 引物	1 μM	4 μL
聚蛋白酶 RVS 引物	1 μM	4 μL
P17 FWD 引物	1 μM	4 μL
P17 RVS 引物	1 μM	4 μL
登革热病毒 FWD 引物	1 μM	4 μL
登革热病毒 RVS 引物	1 μM	4 μL
探针	浓度	添加的体积
聚蛋白酶探针 1	800 nM	4 μL
P17 探针 1	400 nM	4 μL
登革热病毒探针 1	200 nM	4 μL

[0271] 表23.二进制FAM实验6混合液:HIV p17。

[0272]

试剂	浓度	添加的体积
超纯水	-	58 μL
Taq 5x 预混液	-	25 μL
模板	浓度	添加的体积
聚蛋白酶模板	17 nM	-
P17 模板	15 nM	4 μL
登革热病毒模板	17 nM	-
引物	浓度	添加的体积
聚蛋白酶 FWD 引物	1 μM	4 μL
聚蛋白酶 RVS 引物	1 μM	4 μL
P17 FWD 引物	1 μM	4 μL
P17 RVS 引物	1 μM	4 μL
登革热病毒 FWD 引物	1 μM	4 μL
登革热病毒 RVS 引物	1 μM	4 μL
探针	浓度	添加的体积
聚蛋白酶探针 1	800 nM	4 μL

[0273]

P17 探针 1	400 nM	4 μL
登革热病毒探针 1	200 nM	4 μL

[0274] 表24.二进制FAM实验7混合液:HIV聚蛋白酶。

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[0275]

试剂	浓度	添加的体积
超纯水	-	58 μL
Taq 5x 预混液	-	25 μL
模板	浓度	添加的体积
聚蛋白酶模板	17 nM	4 μL
P17 模板	15 nM	-
登革热病毒模板	17 nM	-
引物	浓度	添加的体积
聚蛋白酶 FWD 引物	1 μM	4 μL
聚蛋白酶 RVS 引物	1 μM	4 μL
P17 FWD 引物	1 μM	4 μL
P17 RVS 引物	1 μM	4 μL
登革热病毒 FWD 引物	1 μM	4 μL
登革热病毒 RVS 引物	1 μM	4 μL
探针	浓度	添加的体积
聚蛋白酶探针 1	800 nM	4 μL
P17 探针 1	400 nM	4 μL
登革热病毒探针 1	200 nM	4 μL

[0276] 每个实验的总荧光信号在如上文描述构建的色谱上绘制(图7)。围绕每个阳性对照测量结果的带的宽度是1x测量结果传播的不确定性。该不确定性等于饱和的最后五个PCR循环的荧光信号的标准差。

[0277] 图7显示了该实验的结果。最右边的三个结果显示了单独的登革病毒(DEN)、单独的HIV p17(P17)和HIV聚蛋白酶(TPP)的测量强度。对这些分析物的每个分配的强度如表11所示,且图7确认了三个分析物单独存在时,产生了预期的强度结果(见DEN、P17和TPP)。在测定结束时,基本上所有的探针已被水解,释放了发射信号的基本上所有的荧光团。同定数量的荧光团产生固定的且可预测的信号量。因此,由通过加入到反应体积的探针的数量确定的,存在的目标通常应产生固定量的信号。

[0278] 如预期的,分析物的组合的强度值等于分配的强度之和。因为这种编码方案被设计成非简并的,每个结果可以明确地解码成特定分析物的存在或不存在。例如,从图7的左侧开始,7的结果表明所有三个分析物存在。3的结果表明登革热病毒和HIV p17分析物存在,而HIV聚蛋白酶不存在。5的结果表明登革热病毒和HIV聚蛋白酶存在,而HIV p17不存在。最后,6的结果表明HIV p17和HIV聚蛋白酶存在,而登革热病毒不存在。如在本公开中其他地方描述的,这种编码方案可被扩大以编码额外的分析物。

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序列表

<110> 加州理工学院

<120> 多路生化测定中信号的编码和解码

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<140> PCT/US2013/024509

<141> 2013-02-01

<150> 61/703,093

<151> 2012-09-19

<150> 61/594,480

<151> 2012-02-03

<160> 32

<170> PatentIn version 3.5

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<211> 198

<212> DNA

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aagatggaaa ccaaaaatga tagggggaat tggaggtttt atcaaagtaa gacagtatga 120

tcagatactc atagaaatct gtggacataa agctataggt acagtattag taggacctac 180

acctgtcaac ataattgg 198

<210> 2

<211> 26

<212> DNA

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ccaattatgt tgacaggtgt aggtcc 26

<210> 4

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aagatagagg aagagcaaaa caaaagtaag aaaaaagcac agcaagcagc agctgacaca 180
ggacacagca atcaggtca 199
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aaagcacagc aagcagcagc tga 23
<210> 10
<211> 199
<212> DNA
<213> 恶性疟原虫(Plasmodium falciparum)
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agaaatagct accacatcta aggaaggcag caggcgcgta aattacccaa ttctaaagaa 120
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cgtaaatgct tccctgctgg tgccgatctg ggaccgcgcc gcggagacat tcgagtacca 180

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<220>

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<210> 20
<211> 200
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accgagaagg gcaccttcat catcaacggc accgagcgcg tcgtggtcag ccagctggtc 180
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<400> 23
cgctgttcgt cacggccgag ttcat 25

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<210> 24
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<223> 3'IAbRQSp
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ggagcaggaa cccgacatgt 200
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<211> 22
<212> DNA
<213> 人工序列
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<400> 27
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<210> 28
<211> 24
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<213> 人工序列
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<210> 31
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<223> 人工序列的描述:合成探针
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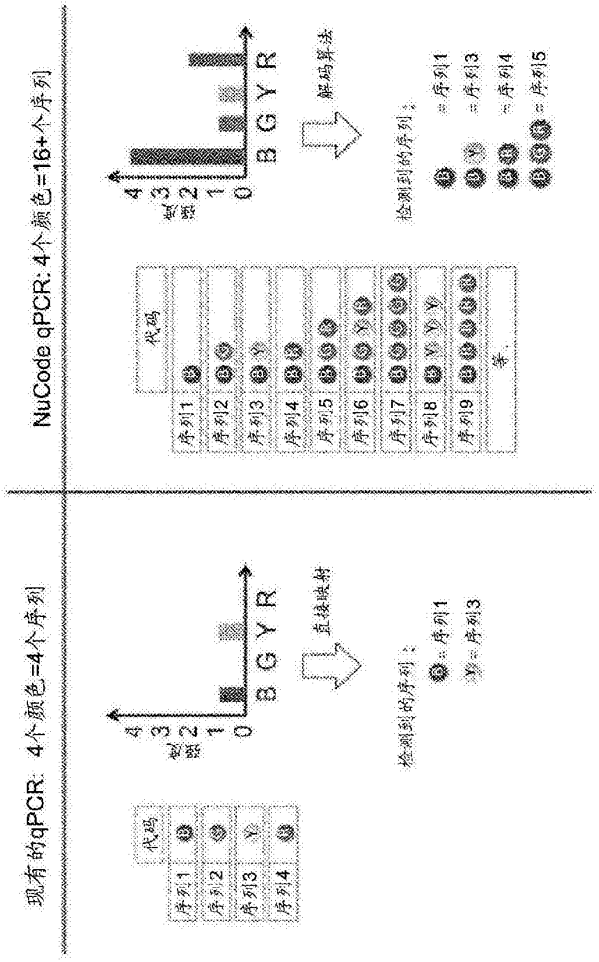


图1

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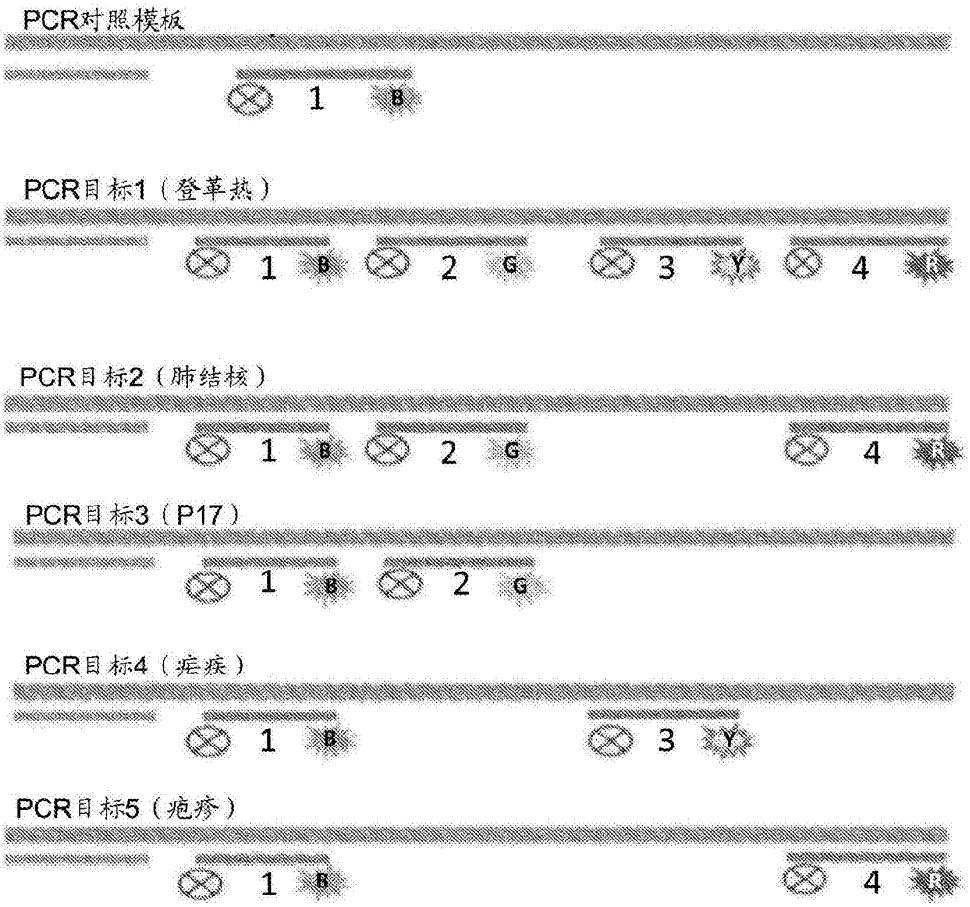


图2

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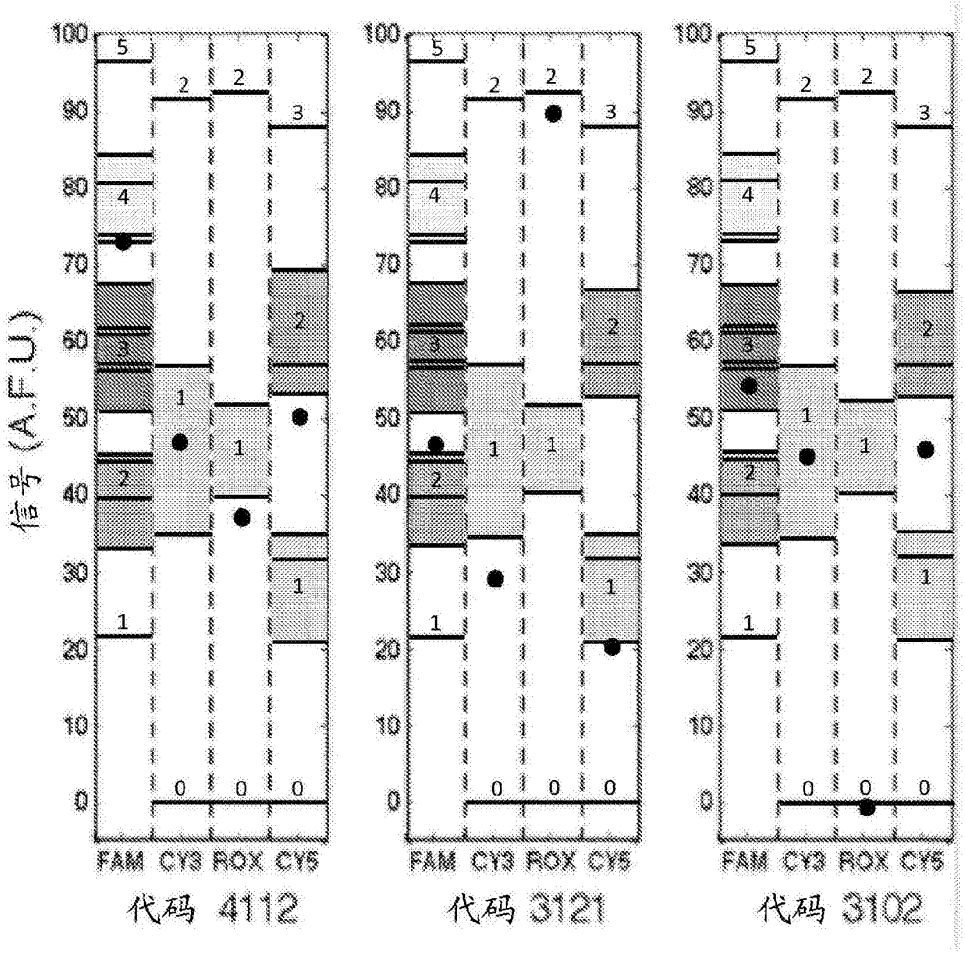


图3

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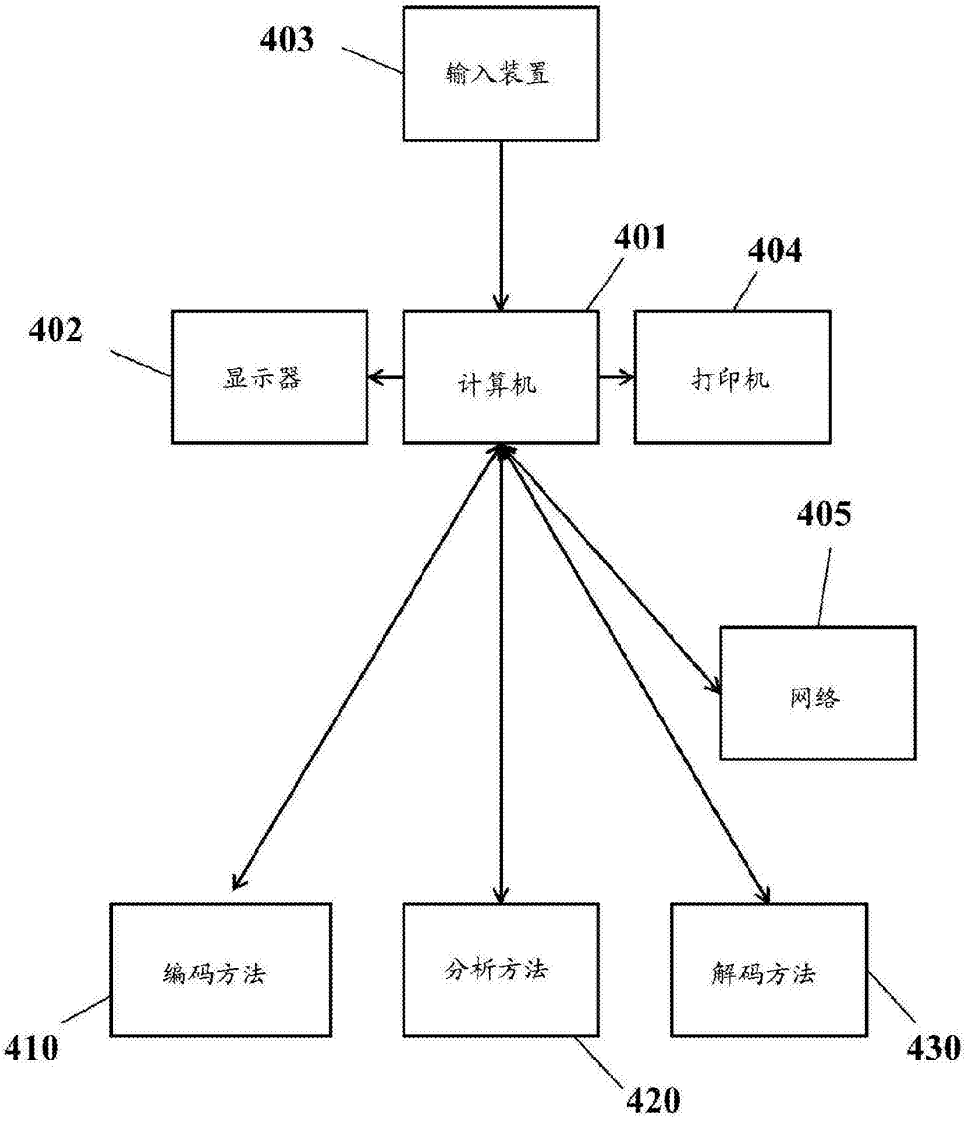


图1

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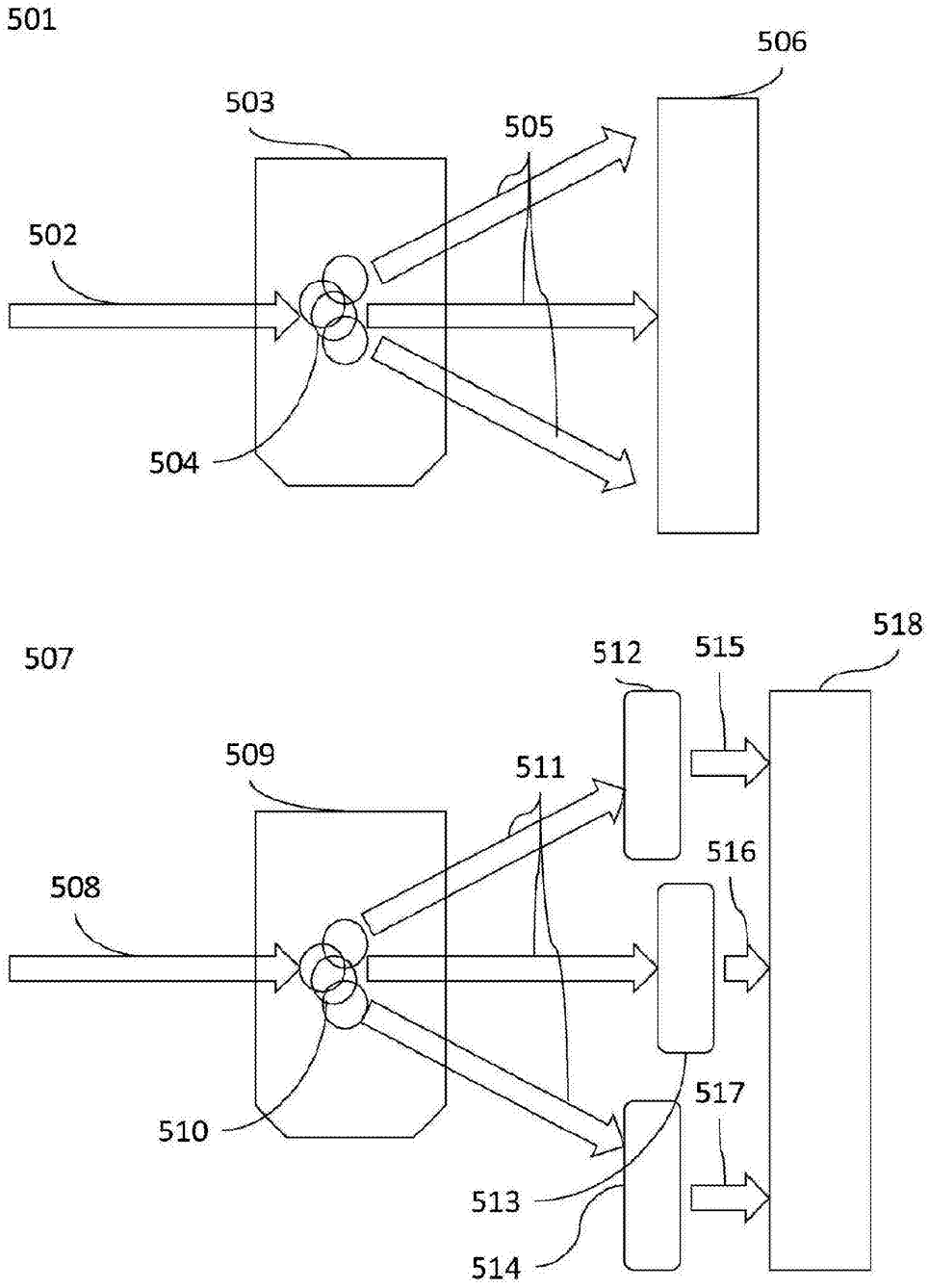


图5

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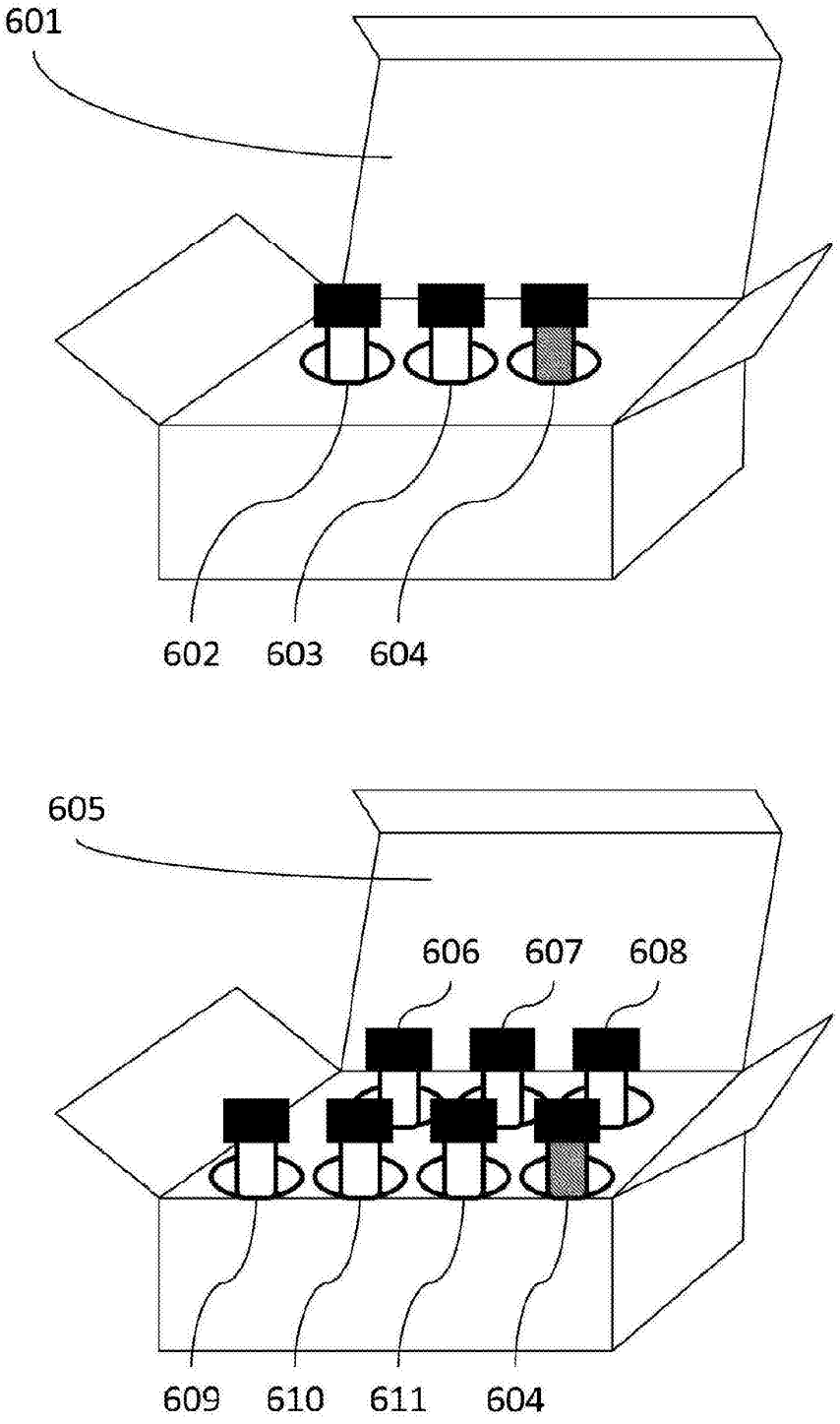


图6

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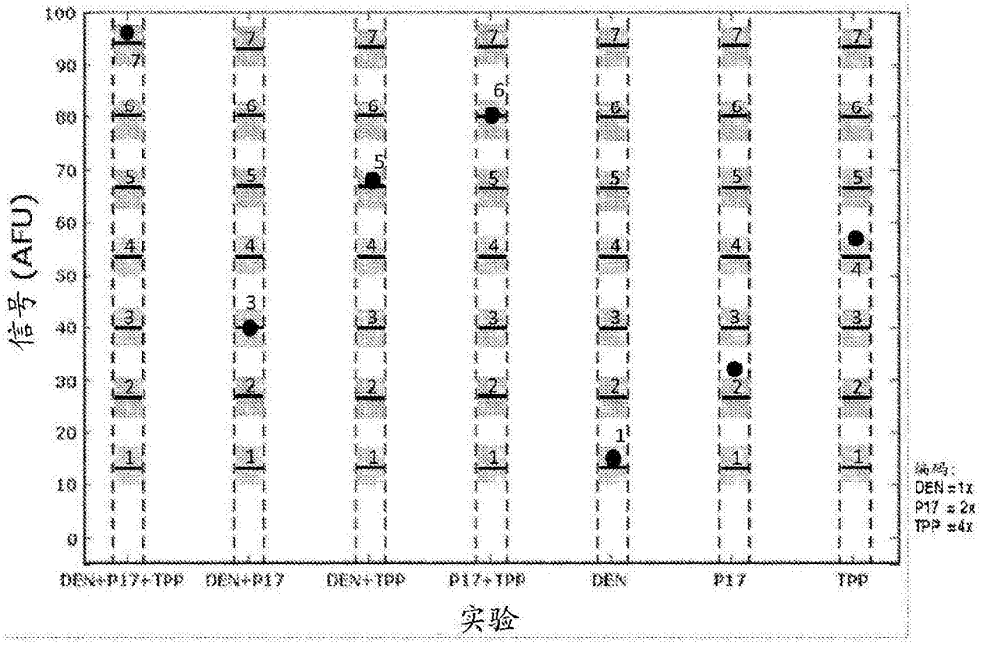
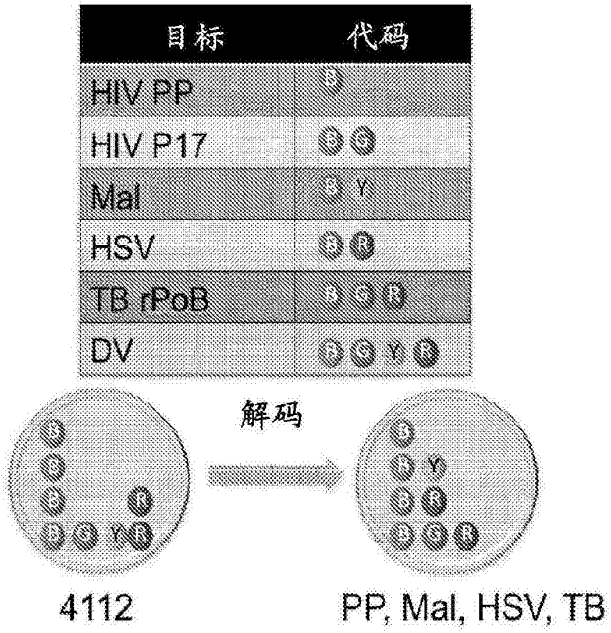


图7

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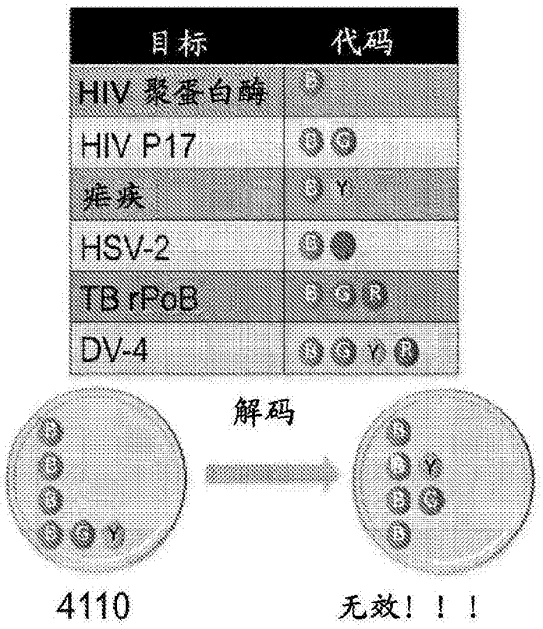


图8

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This translation is machine-generated. It cannot be guaranteed that it is intelligible, accurate, complete, reliable or fit for specific purposes. Critical decisions, such as commercially relevant or financial decisions, should not be based on machine-translation output.

DESCRIPTION CN102150043A

10 Blood transcriptional signature of Mycobacterium tuberculosis infection

[0001]

14 Invention technology field

[0002]

18 The present invention relates generally to the field of Mycobacterium tuberculosis infection, and more particularly to methods for diagnosing, predicting and monitoring latent and active Mycobacterium tuberculosis infection and disease progression before, during and after treatment.

[0003]

24 long form

[0004]

28 This patent application contains long tabular sections.

29 Copies of the forms are available in electronic form from the USPTO website (http://seqdata.uspto.gov/). Electronic copies of this form are available from the USPTO upon request and payment of the fee specified in 37 CFR 1.19(b)(3).

[0005]

35 Background of the invention

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[0006]

39 Without limiting the scope of the invention, it is described in the context of the identification and treatment of Mycobacterium tuberculosis infection.

[0007]

44 Pulmonary tuberculosis (PTB) is a major and growing cause of morbidity and mortality caused by Mycobacterium tuberculosis (M. tuberculosis) worldwide.

46 However, most individuals infected with M. tuberculosis remain asymptomatic, maintaining the infection in a latent form that is thought to be maintained by an active immune response (WHO; Kaufmann, SH & McMichael, AJ., Nat. Med, 2005).

49 This is supported by reports showing that treatment of patients with Crohn's Disease or Rheumatoid Arthritis with anti-TNF antibodies resulted in improvements in autoimmune symptoms. Causes reactivation of TB in patients with prior exposure to M. tuberculosis (Keane).

52 The immune response to M. tuberculosis is multifactorial and includes genetically defined host factors such as TNF and IFN-γ on the Th1 axis and IL-12 (reviewed in Casanova, Ann Rev; Newport). However, immune cells from adult pulmonary TB patients produce IFN-γ, IL-12, and TNF, and IFN-γ therapy does not help improve the disease (reviewed in Reljic, 2007, JInterferon & Cyt Res., 27, 353-63), suggesting that a broader number of host immune factors are involved in protection against M. tuberculosis and in the maintenance of latency. Therefore, knowledge of the host factors induced in latent or active TB can provide information on the immune response capable of controlling M. tuberculosis infection.

[0008]

62 The diagnosis of PTB can be difficult and problematic for a number of reasons.

63 First, demonstrating the presence of typical M. tuberculosis in sputum by microscopic examination (smear positivity) is only 50-70% sensitive, and a positive diagnosis requires isolation of M. tuberculosis by culture, which can take up to 8 weeks. In addition, some patients have sputum that is smear-negative or fail to produce sputum, requiring additional sampling through an invasive procedure such as bronchoscopy. Because of these limitations in the diagnosis of PTB, patients with negative smears are sometimes tested for tuberculin (PPD) skin reaction (Mantoux test). However, tuberculin (PPD) skin reaction cannot differentiate between BCG vaccination, latent or active TB. In response to this problem, assays have been developed that demonstrate immunoreactivity to specific M. tuberculosis antigens that are not present in BCG. However, reactivity to these M. tuberculosis antigens (measured by IFN-γ production by blood cells in the interferon gamma release assay (IGRA)) does not distinguish latent from active disease. Clinically, latent TB is defined by a delayed type of highly sensitive reaction when a patient is injected subcutaneously with PPD and has a positive IGRA result in the absence of clinical symptoms or signs or radiologically active disease. Reactivation of latent/latent tuberculosis (TB) presents serious health hazards with risk of transmission to other individuals, therefore biomarkers reflecting the distinction between latent and active TB patients may be useful in disease control Useful, especially since antimycobacterial drug treatment is difficult and can cause serious side effects.

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[0009]

81 Summary of the invention

[0010]

85 The present invention includes methods and kits for identifying latent versus active tuberculosis (TB) patients (compared to healthy controls).

87 In one embodiment, blood microarray analysis of distinct and reciprocal immune signatures is used to identify, diagnose, track and treat patients with latent and active tuberculosis (TB).

[0011]

92 In one embodiment, the present invention includes methods, systems and kits for distinguishing active and latent Mycobacterium tuberculosis infection in a patient suspected of being infected with Mycobacterium tuberculosis, the method comprising the steps of: A gene expression data set is obtained from whole blood samples; the differential expression of one or more transcribed gene expression modules that distinguish infected patients from uninfected individuals is determined, wherein the data set shows the corresponding Overall changes in polynucleotide levels in one or more transcribed gene expression modules compared to uninfected individuals and based on one or more transcribed gene expression modules that differentiate active from latent infection, distinguish active from latent Mycobacterium tuberculosis (TB) infection.

100 In one aspect, the invention may further include the step of using the determined comparative gene product information to formulate a diagnosis.

[0012]

105 In another aspect, the method may further include the step of using the determined comparative gene product information to plan a prediction, or using the determined comparative gene product information to develop a treatment plan.

108 In an optional aspect, the invention may include the step of distinguishing patients with latent TB from patients with active TB.

110 In one aspect, the modules may include modules M1.2, M1.3, M1.4, M1.5, M1.8, M2.1, M2.4, M2.8, M3.1, M3.2, Data set of genes in M3.3, M3.4, M3.6, M3.7, M3.8 or M3.9 to detect active lung infection.

112 In another aspect, the module may include a data set of genes in modules M1.5, M2.1, M2.6, M2.10, M3.2 or M3.3 to detect latent infection.

114 In yet another aspect, the following genes are downregulated in active lung infection: CD3, CTLA-4, CD28, ZAP-70, IL-7R, CD2, SLAM, CCR7 and GATA-3.

116 In a specific aspect, the module expression profile of Figure 9 is indicative of active lung infection and the module expression profile of Figure 10 is indicative of latent infection.

118 Low expression of genes in modules M3.4, M3.6, M3.7, M3.8 and M3.9 has been found to be indicative of active infection.

120 Overexpression of genes in module M3.1 has also been found to be indicative of active infection.

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[0013]

124 In yet another aspect of the present invention, the method may further comprise the step of distinguishing TB infection from other bacterial infections by measuring gene expression in modules M2.2, M2.3 and M3.5, which are analyzed in In infections other than mycobacteria, it is overexpressed by peripheral blood mononuclear cells or whole blood.

128 Alternatively, the method may include the step of distinguishing differential and reciprocal transcriptional signatures in the blood of latent and active TB patients using two or more of the following modules: M1.3, M1 .4, M1.5, M1.8, M2.1, M2.4, M2.8, M3.1, M3.2, M3.3, M3.4, M3.6, M3.7, M3.8 or M3.9 (for active lung infection) and modules M1.5, M2.1, M2.6, M2.10, M3.2 or M3.3 (for latent infection).

132 Examples of genes upregulated in active pulmonary TB infection compared to healthy patients are selected from Tables 7A, 7D, 7I, 7J and 7K.

134 Further examples of genes downregulated in active pulmonary TB infection compared to healthy patients are selected from Tables 7B, 7C, 7E, 7F, 7G, 7H, 7L, 7M, 7N, 7O and 7P.

136 In a specific aspect, genes upregulated in latent TB infection relative to healthy patients can be selected from Table 8B.

138 In another specific aspect, genes that are downregulated in latent TB infection relative to healthy patients can be selected from Tables 8A, 8C, 8D, 8E, and 8F.

[0014]

143 Another embodiment of the present invention is a method of distinguishing active from latent Mycobacterium tuberculosis infection in a patient suspected of being infected with Mycobacterium tuberculosis, said method comprising the steps of: A first gene expression data set was obtained from a first clinical group of infected individuals, a second gene expression data set was obtained from a clinical group of patients with latent M. tuberculosis infection, and a third gene expression data set was obtained from a clinical group of uninfected individuals. Expression profiling; generating a gene cluster data set containing differential expression of genes between any two of the first, second, and third data sets; and determining a unique pattern of expression/expression, the pattern indicating Latent infection, active infection or health.

151 In one aspect, each clinical group is assigned to a unique expression/performance profile for each of the 119 genes in Table 6.

153 In another aspect, the values of the first and third data sets are compared and the values of the data set from the third data set are subtracted therefrom.

155 In another specific aspect, values from the second and third data sets are compared and values from the third data set are subtracted therefrom.

157 In a specific embodiment, the method may further comprise the step of comparing the values of two different data sets and subtracting the values of the remaining data sets, thereby distinguishing between patients with latent infection and patients with active infection. infected patients as well as uninfected individuals.

160 In one aspect, the invention may further comprise the step of using the determined comparative gene product information to formulate a diagnosis or prediction.

162 In yet another aspect, the method includes the step of using the determined comparative gene product information to develop a treatment plan.

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164 The method may also include the step of differentiating patients with latent TB from patients with active TB by analyzing expression/expression in genes and patient clusters.

[0015]

169 In a specific aspect, the method may further comprise the step of determining the expression levels of the following genes: ST3GAL6, PAD14, TNFRSF12A, VAMP3, BR13, RGS19, PILRA, NCF1, LOC652616, PLAUR (CD87), SIGLEC5, B3GALT7, IBRDC3 (NKLAM), ALOX5AP(FLAP), MMP9, ANPEP(APN), NALP12, CSF2RA, IL6R(CD126), RASGRP4, TNFSF14(CD258), NCF4, HK2, ARID3A, PGLYRP1(PGRP), which are low in latent TB patients. Expression/low expression, but not in the blood of healthy individuals or patients with active TB.

175 In another specific aspect, the method may further comprise the step of determining the expression level of the genes: ABCG1, SREBF1, RBP7 (CRBP4), C22orf5, FAM101B, S100P, LOC649377, UBTD1, PSTPIP-1, RENBP, PGM2, SULF2, FAM7A1, HOM-TES-103, NDUFAF1, CES1, CYP27A1, FLJ33641, GPR177, MID1IP1(MIG-12), PSD4, SF3A1, NOV(CCN3), SGK(SGK1), CDK5R1, LOC642035, which in healthy controls Overexpressed/overrepresented in the blood of individuals, but underexpressed/underrepresented in the blood of latent TB patients, and underexpressed/underrepresented in the blood of active TB patients.

181 In another specific aspect, the method may further comprise the step of determining the expression levels of ARSG, LOC284757, MDM4, CRNKL1, IL8, LOC389541, CD300LB, NIN, PHKG2, HIP1 in the blood of healthy individuals. Expressed/over-expressed, under-expressed/under-expressed in latent and active TB patients.

185 In a specific aspect, the method may further comprise the step of determining the expression levels of the following genes: PSMB8 (LMP7), APOL6, GBP2, GBP5, GBP4, ATF3, GCH1, VAMP5, WARS, LIMK1, NPC2, IL-15, LMTK2, STX11 (FHL4), which is overexpressed/overrepresented in the blood of active TB and underexpressed/underrepresented in the blood of latent TB patients and healthy control individuals.

189 In a specific aspect, the method may further comprise the step of determining the expression levels of the following genes: FLJ11259 (DRAM), JAK2, GSDMDC1 (DF5L) (FKSG10), SIPAIL1, [2680400] (KIAA1632), ACTA2 (ACTSA), KCNMB1 (SLO-BETA), which is overexpressed/overrepresented in blood from active TB patients and underexpressed/underrepresented in blood from latent TB patients and healthy control individuals.

194 In a specific aspect, the method may further comprise the step of determining the expression levels of the following genes: SPTANI, KIAAD179(Nnp1)(RRP1), FAM84B(NSE2), SELM, IL27RA, MRPS34, [6940246](IL23A), PRKCA (PKCA), CCDC41, CD52(CDW52), [3890241](ZN404), MCCC1(MCCA/B), SOX8, SYNJ2, FLJ21127, FHIT, which have low expression/low expression in the blood of active TB patients, while This was not the case in the blood of latent TB patients or healthy controls.

199 In a specific aspect, the method may further comprise the step of determining the expression levels of the following genes: CDKL1 (p42), MICALCL, MBNL3, RHD, ST7 (RAY1), PPR3R1, [360739] (PIP5K2A), AMFR, FLJ22471, CRAT(CAT1), PLA2G4C, ACOT7(ACT)(ACH1), RNF182, KLRC3(NKG2E), HLA-DPB1, which have low expression/low expression in the blood of healthy control individuals and pass in the blood of latent TB patients Expression/overexpression, and overexpression/overexpression in the blood of active TB patients.

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[0016]

208 Yet another embodiment of the present invention is a method for distinguishing active from latent Mycobacterium tuberculosis infection in a patient with suspected Mycobacterium tuberculosis infection, said method comprising the steps of: obtaining from a whole blood sample Gene expression data sets; classifying the gene expression data sets into one or more transcribed gene expression modules; and identifying the features of the one or more transcribed gene expression modules that differentiate between active and latent Mycobacterium tuberculosis infections. Differential expression was mapped to differentiate between active and latent M. tuberculosis infection.

215 In one aspect, the data set includes TRIM genes.

216 In one aspect, the data set includes TRIM genes, specifically TRIM5, 6, 19 (PML), 21, 22, 25, 68, overexpressed/expressed in active pulmonary TB.

218 In one aspect, the dataset of TRIM genes, including TRIM 28, 32, 51, 52, 68, are low expressed/expressed in active pulmonary TB.

[0017]

223 Another embodiment of the invention is a method of diagnosing active and latent Mycobacterium tuberculosis infection in a patient suspected of being infected with Mycobacterium tuberculosis, said method comprising: detecting one or more Differential expression of a transcribed gene expression module that differentiates between infected and uninfected patients, wherein whole blood demonstrates expression of one or more transcribed gene expression modules in said one or more transcribed gene expression modules compared to corresponding uninfected patients. Global changes in polynucleotide levels that differentiate between active and latent Mycobacterium tuberculosis infection.

230 In another aspect, the method includes one or more of the steps of using the determined comparative gene product information to formulate a diagnosis, using the determined comparative gene product information to formulate a diagnosis, and using the determined comparative gene product information. Information on steps to develop a treatment plan.

234 In an optional aspect, the method may include the step of distinguishing patients with latent TB from active TB.

236 In one aspect, the modules may include gene data sets in the following modules: M1.2, M1.3, M1.4, M1.5, M1.8, M2.1, M2.4, M2.8, M3.1, M3.2, M3.3, M3.4, M3.6, M3.7, M3.8 or M3.9 to detect active lung infection.

239 In another aspect, the module may include genetic data sets in the following modules: M1.5, M2.1, M2.6, M2.10, M3.2, or M3.3, thereby detecting latent infection.

241 In yet another aspect, the following genes are downregulated in active lung infection, CD3, CTLA-4, CD28, ZAP-70, IL-7R, CD2, SLAM, CCR7 and GATA-3.

243 In one specific aspect, the expression profile of the module in Figure 9 is indicative of active lung infection and the expression profile of the module in Figure 10 is indicative of latent infection.

245 Low expression of genes in modules M3.4, M3.6, M3.7, M3.8 and M3.9 has been found to be indicative of active infection.

247 Overexpression of genes in module M3.1 was also found to be indicative of active infection.

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[0018]

251 In yet another aspect of the invention, the method further includes the step of distinguishing TB infection from other bacterial infections by measuring gene expression in modules M2.2, M2.3 and M3.5, said modules being present in Mycobacterium In other infections, it is overexpressed by peripheral blood mononuclear cells or whole blood.

255 Optionally, the method may include the step of distinguishing different and reciprocal transcriptional signatures in the blood of latent and active TB patients using two or more of the following modules: for active lung infection, M1.3, M1.4, M1.5, M1.8, M2.1, M2.4, M2.8, M3.1, M3.2, M3.3, M3.4, M3.6, M3. 7. M3.8 or M3.9. For latent infection, it is module M1.5, M2.1, M2.6, M2.10, M3.2 or M3.3.

259 Examples of genes upregulated in active pulmonary TB infection relative to healthy patients are selected from Tables 7A, 7D, 7I, 7J and 7K.

261 Other examples of genes downregulated in active pulmonary TB infection relative to healthy patients are selected from Tables 7B, 7C, 7E, 7F, 7G, 7H, 7L, 7M, 7N, 7O and 7P.

263 In a specific aspect, examples of genes upregulated in latent TB infection relative to healthy patients are selected from Table 8B.

265 In another specific aspect, examples of genes downregulated in latent TB infection relative to healthy patients are selected from Tables 8A, 8C, 8D, 8E, and 8F.

[0019]

270 Another embodiment of the invention is a kit for diagnosing active and latent Mycobacterium tuberculosis infection in a patient suspected of being infected with Mycobacterium tuberculosis, the kit comprising: a gene expression detector that obtains a gene expression data set; and a processor capable of comparing gene expression obtained from whole blood to a preset gene module data set that distinguishes between infected and uninfected patients, wherein the whole blood Blood demonstrates global changes in polynucleotide levels in one or more transcribed gene expression modules compared to corresponding uninfected patients, thereby distinguishing active from latent M. tuberculosis infection.

[0020]

280 Yet another embodiment includes a system for diagnosing patients with active and latent Mycobacterium tuberculosis infection, comprising: a gene expression data set from said patient; and capable of comparing gene expression obtained from whole blood with a preset A processor that compares gene module data sets that differentiate between infected and uninfected patients, wherein whole blood demonstrates expression of one or more transcribed genes in one or more transcribed gene expression modules compared to corresponding uninfected patients. Overall changes in polynucleotide levels to differentiate between active and latent Mycobacterium tuberculosis infection, wherein for active lung infection, the module is selected from the group consisting of M1.3, M1.4, M1.5, M1.8, M2.1, M2.4, M2.8, M3.1, M3.2, M3.3, M3.4, M3.6, M3.7, M3.8 or M3.9, for latent For sexual infections, select from modules M1.5, M2.1, M2.6, M2.10, M3.2 or M3.3.

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[0021]

293 Brief description of the drawings

[0022]

297 For a more complete understanding of the features and advantages of the present invention, reference is made to the detailed description of the invention and the accompanying drawings, in which:

[0023]

302 Figure 1 shows gene array expression from 42 participants. Genes present in at least 2 samples (PLA2), genes up- or down-regulated 2-fold compared to the median number, were clustered by Pearson correlation coefficients to compare activity. PTB, latent TB, healthy BCG unvaccinated controls and healthy BCG vaccinated controls;

[0024]

309 Figure 2 shows gene array expression results obtained from PAL2, up- or down-regulated expression by 2-fold, screened for statistically significant differences in expression between clinical groups using a non-parametric test (Kruskal- Wallis), $P < 0.01$, and using the Benjamini-Hochberg correlation coefficient (1473 genes) and pooled independently using the Pearson correlation coefficient to compare active PTB, latent TB, and healthy controls;

[0025]

317 Figures 3A-3D show gene array expression results from PAL2, 2-fold up- or down-regulated, screened for statistically significant differences in expression between clinical groups using a non-parametric test (Kruskal-Wallis), $P < 0.01$, and using the Benjamini-Hochberg correction and subsequently filtered for the presence of the Gene Ontology term for the biological process "immune response" in gene annotations and independently clustered using the Pearson correlation coefficient (158 genes).

322 These 158 genes are shown in 4 figures for ease of viewing (Figures 3A-3D).

[0026]

326 Figure 3A shows the gene array expression results comparing active PTB, latent TB, unvaccinated control of healthy BCG, and vaccinated control of healthy BCG;

[0027]

331 Figure 3B shows the gene array expression results comparing active PTB, latent TB, unvaccinated control of

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healthy BCG, and vaccinated control of healthy BCG;

[0028]

336 Figure 3C shows the gene array expression results comparing active PTB, latent TB, unvaccinated control of healthy BCG, and vaccinated control of healthy BCG;

[0029]

341 Figure 3D shows the gene array expression results comparing active PTB, latent TB, unvaccinated control of healthy BCG, and vaccinated control of healthy BCG;

[0030]

346 Figure 4 shows the gene array expression results from 42 participants, genes present in at least 2 samples (PAL2), genes 2-fold higher or lower than the median, selected by the Pearson correlation coefficient set As TRIM genes, active PTB, latent TB, unvaccinated control of healthy BCG and vaccinated control of healthy BCG were compared;

[0031]

353 Figure 5A shows details of gene array expression results from 42 participants, genes present in at least 2 samples (PAL2), genes 2-fold higher or lower than the median, clustered by Pearson correlation coefficient, compared active PTB, latent TB, healthy BCG unvaccinated controls and healthy BCG vaccinated controls, showing the inhibitory immune regulatory ligands (PDL1/CD274, PDL2/CD273) in active TB patients Medium and over-expressed.

[0032]

361 Figure 5B shows unfiltered gene array expression results, which confirm that PDL1 is expressed only in active TB patients.

[0033]

366 Figure 6 shows the screening of genes present in at least 2 samples in the gene array expression results, which are up-regulated or down-regulated 2 times compared with the median, and have statistically significant differences in expression between groups ($P<0.1$, with Kruskal-Wallis nonfactorial test of Bonferroni correlation coefficient) (46 genes) in which active PTB, latent TB, healthy BCG unvaccinated controls, and healthy BCG were compared independently using Pearson correlation Inoculated controls;

[0034]

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374 Figure 7 shows the screening of genes present in at least 2 samples in the gene array expression results, which "appear" to be up-regulated or down-regulated 2 times compared with the median, and have statistically significant differences in expression between groups (P< 0.05, Kruskal-Wallis nonfactorial test with Bonferroni correlation coefficient) (18 genes), in which active PTB, latent TB, healthy BCG unvaccinated controls and Vaccinated control of healthy BCG;

[0035]

382 Figure 8A shows the results of combining different statistical screens to distinguish all 3 clinical groups, which were applied to screen for a list of genes present in at least 2 samples that "appeared" to be up-regulated or down-regulated compared to the median 2 times.

385 The transcripts shown are statistically significantly differently expressed between latent and healthy (P<0.005, Wilcoxon-Mann-Whitney non-parametric test without correlation coefficient), and between active and healthy, Transcripts were statistically significantly differentially expressed (P<0.5, Wilcoxon-Mann-Whitney non-parametric test with Bonferroni correlation coefficient) - a total of 119 genes (cohort representation of patients/clinical groups) independently centered using Pearson correlation coefficient (horizontally, groups of genes are represented vertically); these 119 genes are shown separately in 5 further figures (Figures 8B-8F) to facilitate viewing and to show that subgroups of these genes may also be used to differentiate Different clinical groups (i.e. active, latent and healthy).

[0036]

396 Figure 8B shows gene array expression results filtered for genes present in at least 2 samples that "appear" to be up-regulated or down-regulated 2-fold compared to the median, transcripts statistically expressed in latent and healthy Significantly different (P<0.005, Wilcoxon-Mann-Whitney non-factorial test without correlation coefficient), and transcripts were statistically significantly differently expressed between active and healthy (P<0.5, Wilcoxon with Bonferroni correlation coefficient - Mann-Whitney nonparametric test) (groups of patients/clinical groups are represented horizontally, groups of genes are represented vertically);

[0037]

405 Figure 8C shows gene array expression results filtered for genes present in at least 2 samples that "appear" to be up-regulated or down-regulated 2-fold compared to the median, transcripts statistically expressed in latent and healthy Significantly different (P<0.005, Wilcoxon-Mann-Whitney non-factorial test without correlation coefficient), and transcripts were statistically significantly differently expressed between active and healthy (P<0.5, Wilcoxon with Bonferroni correlation coefficient - Mann-Whitney non-parametric test);

[0038]

413 Figure 8D shows gene array expression results filtered for genes present in at least 2 samples that "appear" to be up-regulated or down-regulated 2-fold compared to the median, transcripts statistically expressed in latent and healthy Significantly different (P<0.005, Wilcoxon-Mann-Whitney non-factorial test without correlation

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coefficient), and transcripts were statistically significantly differently expressed between active and healthy (P<0.5, Wilcoxon with Bonferroni correlation coefficient - Mann-Whitney nonparametric test) Groups of patients/clinical groups are represented horizontally, groups of genes are represented vertically);

[0039]

422 Figure 8E shows gene array expression results filtered for genes present in at least 2 samples that "appear" to be up-regulated or down-regulated 2-fold compared to the median, transcripts statistically expressed in latent and healthy Significantly different (P<0.005, Wilcoxon-Mann-Whitney non-factorial test without correlation coefficient), and transcripts were statistically significantly differently expressed between active and healthy (P<0.5, with Bonferroni correlation coefficient Wilcoxon-Mann-Whitney non-parametric test) (groups of patients/clinical groups are represented horizontally, groups of genes are represented vertically);

[0040]

431 Figure 8F shows that gene array expression results were screened for genes present in at least 2 samples that were up- or down-regulated 2-fold compared to the median, transcripts with statistically significant differences in latent and healthy expression (P <0.005, Wilcoxon-Mann-Whitney non-factorial test without correlation coefficient), and transcripts were statistically significantly differently expressed between active and healthy (P<0.5, Wilcoxon-Mann-Whitney with Bonferroni correlation coefficient Non-parametric test) (groups of patients/clinical groups are represented horizontally, groups of genes are represented vertically);

[0041]

440 Figure 9 shows gene array expression results from gene module analysis of PTB (9) versus control (6): from 5281 genes, screening for PAL2, by Wilcoxon-Mann-Whitney test, $p < 0.05$ and without multiple testing calibration , active PTB and healthy controls are expressed statistically significantly differently; and

[0042]

446 Figure 10 shows gene array expression results from gene module analysis of PTB (9) versus control (6): from 3137 genes, screened for PAL2, by Wilcoxon-Mann-Whitney test, $p < 0.05$ and without multiple testing calibration. , active PTB and healthy controls were expressed statistically significantly differently.

[0043]

452 Detailed description of the invention

[0044]

456 While the preparation and use of various embodiments of the invention are discussed in detail below, it should be understood that the invention provides a wide variety of practical inventive concepts that can be

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embodied in a wide variety of specific contexts.

459 The specific embodiments discussed herein are merely illustrative of specific ways to make and use the invention and do not limit the scope of the invention.

[0045]

464 To facilitate understanding of the present invention, a number of terms are defined below.

465 The terms defined herein have the meanings commonly understood by those of ordinary skill in the art relevant to the present invention.

467 Terms such as "a/an", "an" and "the" are not intended to refer only to singular entities, but include a general class of which specific instances may be used as illustrations.

469 The term is used herein to describe specific embodiments of the invention, however its use does not limit the invention except as otherwise indicated in the claims.

471 Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

473 The following references provide the skilled person with general definitions of various terms used in this invention: Singleton et al., Dictionary of Microbiology and Molecular Biology (2d ed. 1994); The Cambridge Dictionary of Science and Technology (Walker ed., 1988); The Glossary of Genetics, 5TH ED., R. Rieger et al. (eds.), Springer Verlag (1991); and Hale & Marham, The Harper Collins Dictionary of Biology (1991).

[0046]

480 Different biochemical and molecular biological methods are known in the art.

481 For example, methods for isolating and purifying nucleic acids are described in detail in WO 97/10365; WO 97/27317; Laboratory Techniques in Biochemistry and Molecular Biology: Hybridization with Nucleic Acid Probes, Part I. Theory and Nucleic Acid Preparation, (P. Tijssen, ed.

484) Chapter 3 of Elsevier, N.Y. (1993); Sambrook et al., Molecular Cloning: ALaboratory Manual, Cold Spring Harbor Press, N.Y., (1989); and Current Protocols in Molecular Biology, (Ausubel, F.M. et al., eds.

486) John Wiley & Sons, Inc., New York (1987-1999), including appendices.

[0047]

490 bioinformatics definition

[0048]

494 As used herein, "object" refers to any target item or information (generally textual, including nouns, verbs, adjectives, adverbs, phrases, sentences, symbols, numeric symbols, etc.).

496 Therefore, an object is anything that can form a relationship, and anything that can be obtained, authenticated and/or searched from the source.

498 "Objects" include but are not limited to target entities, such as genes, proteins, diseases, phenotypes, mechanisms, drugs, etc.

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500 In some aspects, the objects may be data, as described further below.

[0049]

504 As used herein, a "relationship" refers to objects that co-occur in the same unit (such as a phrase, a sentence, two or more lines of text, a paragraph, a portion of a web page, a page, a magazine, a paper, a book, etc.).

506 It may be text, symbols, numbers, and combinations thereof.

[0050]

510 As used herein, "metadata content" refers to information organized according to text in a data source.

511 Metadata may include standard metadata (such as Dublin Core metadata) or may be specimen-specific.

512 Examples of metadata formats include, but are not limited to, Machine Readable Catalog (MARC) records for library catalogs, Source Description Format (RDF), and Extensible Markup Language (XML).

514 Meta-objects may be generated manually or through automated information extraction algorithms.

[0051]

518 As used herein, an "engine" refers to a program that implements the core or critical functionality of other programs.

520 For example, an engine can be a central program in an operating system or application program that regulates the overall operation of other programs.

522 The term "engine" may also refer to a program that includes algorithms that can be changed.

523 For example, a knowledge discovery engine may be designed so that the way it determines relationships may change to reflect new rules for determining and ordering relationships.

[0052]

528 As used herein, "semantic analysis" refers to the determination of relationships between words representing similar concepts, such as by removing suffixes or adding parts or by employing a thesaurus.

530 "Statistical analysis" refers to techniques based on counting the number of occurrences of each item (word, root, stem, metagram, phrase, etc.).

532 In a collection of unqualified objects, the same phrase used in different contexts may represent different concepts.

534 Statistical analysis of phrase co-occurrences may help analyze semantic ambiguity.

535 "Syntactic analysis" can be used to further reduce ambiguity through part-of-speech analysis.

536 As used herein, one or more such analyzes are more generally referred to as "lexical analysis."

537 "Artificial intelligence (AI)" refers to methods by which non-human devices, such as computers, perform tasks that humans would consider noteworthy or "intelligent."

539 Examples include identifying pictures, understanding spoken words or written words, and solving problems.

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[0053]

543 Terms such as "data", "dataset" and "information" are often used interchangeably, as are "information" and "knowledge".

545 As used herein, "data" is the most basic unit, which is a measurement or collection of measurements based on an experiment.

547 Data is collected to form information, but it is essentially independent of this and can be combined into a dataset, a collection of data.

549 In contrast, information is obtained from the target, such that data (units) may be collected on race, gender, height, weight, and diet for the purpose of discovering variables associated with cardiovascular disease risk.

551 However, the same data might be used to develop recipes or generate "information" about dietary preferences, i.e. the likelihood that a particular product will have a higher likelihood of being sold in a supermarket.

[0054]

556 As used herein, the term "database" is a repository of original or collected data, or even different aspects of information found in the data field.

558 A database may contain one or more data sets.

559 A database is generally organized so that its contents can be accessed, managed, and updated (eg, the database is dynamic).

561 The terms "database" and "source" may also be used interchangeably in this disclosure since the original source of data and information is a database.

563 However, "source database" or "source data" generally refers to data, such as unstructured text and/or structured data that is input into the system for identifying objects and determining relationships.

565 The source database may or may not be a relational database.

566 However, system databases typically include relational databases, or some equivalent type of database, which store values related to relationships between objects.

[0055]

571 As used herein, "system database" and "relational database" are used interchangeably and refer to one or more collections of data organized as a set of tables containing data in which the data is combined in a predetermined kind of.

574 For example, a database table may include one or more categories set by a column (eg, attribute), and a database row may include objects unique to the category set by a column.

576 Thus, a property of an object such as a gene may have columns for its presence, absence, and/or expression level of that gene.

578 A row in a relational database may also be referred to as "a set" and is usually defined by the numerical values of its columns.

580 A "field" in the context of a relational database is a range of valid values, such as the fields a column may include.

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[0056]

585 As used herein, "knowledge domain" refers to the area of study in which the system is operational, e.g., all biomedical data.

587 It should be pointed out that it is beneficial to combine data from several domains, for example, biomedical data and engineering data, because the different data can sometimes be incomprehensible to ordinary people who are only familiar with one field or exploration/research (one domain). Connect things that go together.

590 "Distributed database" means a database that may be distributed or replicated at various points on a network.

[0057]

594 As used herein, "information" refers to a collection of data, which may include numbers, letters, a collection of numbers, a collection of letters, or a conclusion resulting from or derived from a collection of data.

596 "Data" is a measurement or statistical quantity and is the basic unit of information.

597 "Information" may also include other types of data, such as words, symbols, text, such as unstructured free text, coding, etc.

599 "Knowledge" is loosely defined as a collection of information that provides sufficient understanding of a system to model causes and effects.

601 To extend the previous example, information on demographic characteristics, gender, and previous purchases may be used to develop regional marketing strategies for food sales, while information on nationality may be used by buyers as guidelines for product imports.

604 It is important to point out that there are no strict boundaries between data, information and knowledge; the three terms are sometimes considered equivalent.

606 Generally, data comes from testing, information comes from correlation, and knowledge comes from modeling.

[0058]

611 As used herein, a "program" or "computer program" generally refers to a syntactic unit that conforms to the rules of a particular programming language and includes declaration files and statements or instructions, which may be divided into "code segments" that are required to solve a problem, or perform specific functions, tasks, and questions.

615 A programming language is generally an artificial language used to express programs.

[0059]

619 As used herein, "system" or "computer system" refers generally to one or more computers, peripheral devices, and software that performs data processing.

621 "Users" or "system operators" generally include people who use computer networks for data processing and information exchange through "user equipment" (such as computers, wireless devices, etc.).

623 A "computer" is generally a functional unit capable of performing substantial calculations, including a variety of arithmetic operations and logical operations without human intervention.

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[0060]

628 As used herein, "application software" or "application program" generally refers to software or programs that are specific to solving application problems.

630 "Application problems" are typically submitted by end users, the resolution of which requires information processing.

[0061]

635 As used herein, "natural language" refers to a language whose rules are based on existing usage and are not specifically prescribed, such as English, Spanish, or Chinese.

637 As used herein, "artificial language" refers to a language whose rules are explicitly established prior to its use, such as a computer programming language such as C, C++, JAVA, BASIC, FORTRAN, or COBOL.

[0062]

642 As used herein, "statistical correlation" refers to the use of one or more ranking schemes (O/E ratio, strength, etc.) in which a relationship is determined to be statistically relevant if it occurs significantly more frequently than would be expected by random chance of.

[0063]

648 As used herein, the terms "co-regulated genes" or "transcriptional modules" are used interchangeably to refer to a set of gene expression profiles (eg, signal values associated with a specific gene sequence) of a specific gene.

651 Each transcription module establishes a link between two key pieces of data, namely the literature search part and the numerical gene expression data from actual experiments on gene microarrays.

653 Gene sets selected into transcription modules are based on analysis of gene expression data (module selection algorithm described above).

655 Additional steps were taught by Chaussabel, D. & Sher, A. Mining microarray expression data by literature profiling.

657 Genome Biol 3, RESEARCH0055(2002), (http://genomebiology.com/2002/3/10/research/0055) (the relevant portions are incorporated herein by reference) and expression data derived from the disease or condition of interest, the diseases or symptoms such as systemic lupus erythematosus, arthritis, lymphoma, cancer, melanoma, acute infection, autoimmune diseases, autoinflammatory diseases, etc.

[0064]

664 Examples of keywords used to derive part of the literature search or contribute to the transcription module are listed in the table below.

666 The skilled person should be aware that other terms can readily be selected in other contexts, such as specific cancers, specific infectious diseases, transplantation, etc.

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668 For example, genes and signals for those involved in T-cell activation are described below with the module ID "M 2.8", where specific keywords (e.g., lymphoma, T-cell, CD4, CD8, TCR, thymus, lymphoid, IL2) was used to identify key T-cell related genes, such as T-cell surface markers (CD5, CD6, CD7, CD26, CD28, CD96); molecules expressed by lymphoid lineage cells (lymphotoxin beta, IL2-induced T cell kinase, TCF7; and T cell differentiation protein mal, GATA3, STAT5B).

673 The entire module then generates a transcriptional module by linking data from patient populations for these genes regardless of platform, presence/absence and/or up- or down-regulation.

675 In some cases, the gene expression profiles did not match (at this time) any specific gene clusters and data for these disease symptoms, yet specific physiological pathways (e.g., cAMP signaling, zinc finger proteins, cell surface markers, etc.).

678 Indeed, a gene expression data set may be used to extract genes with coordinated expression before matching with a keyword search, i.e. any data set may be correlated with each other before being cross-referenced with a second data set.

[0065]

684 Table 1.

685 Transcription module

[0071]

689 biological definition

[0072]

693 As used herein, the term "array" refers to a solid support or matrix having one or more peptides or nucleic acid probes attached to the support.

695 Arrays typically have one or more different nucleic acid or peptide probes coupled to different known locations on the surface of the substrate.

697 These arrays, also called "microarrays" or "gene chips," may have 10,000; 20,000; 30,000 or 40,000 different identifiable genes based on a known genome (such as the human genome).

699 These arrays are used to detect the entire "transcriptome" or transcript pool of genes expressed or found in a sample, e.g., nucleic acids expressed as RNA, mRNA, etc., which may be subjected to RT and/or RT-PCR, resulting in DNA replicons of complementary sets.

702 Arrays can be prepared using mechanical synthesis methods, light-induced synthesis methods, and other methods combined with non-photolithographic and/or photolithographic methods and solid-phase synthesis methods.

[0073]

708 Different techniques for the synthesis of these nucleic acid arrays are described, such as fabrication on virtually arbitrarily shaped surfaces or even multiple surfaces.

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710 The array may be of peptides or nucleic acids on beads, gels, polymeric surfaces, fibers such as optical fibers, glass, or any other suitable matrix.

712 The array may be assembled in such a manner that diagnostics or other operations of the entire included device may be performed, see, for example, U.S. Patent No. 6,955,788, the relevant portions of which are incorporated herein by reference.

[0074]

718 As used herein, the term "disease" refers to a physiological state of any abnormal biological state of cells of an organism.

720 Diseases include, but are not limited to, interruption, cessation or disorder of cells, tissues, body functions, systems or organs, which may be intrinsic, genetic, caused by infection, abnormal cell function, abnormal cell division, etc.

723 Diseases causing "disease states" are generally harmful to the biological system, ie the host of the disease.

724 With regard to the present invention, any biological condition, such as infection (such as viruses, bacteria, fungi, parasites, etc.), inflammation, autoinflammation, autoimmunity, allergic reaction, allergy, precancerous lesions, malignancy, surgery, transplantation, physiological, etc. related to diseases or disorders are considered disease states.

728 Pathological conditions are often equated with disease states.

[0075]

732 Disease states may also be classified into different levels of disease states.

733 As used herein, grade of disease or disease state is a subjective measure that reflects the progression of the disease or disease state, as well as physiological responses before, during, and after treatment.

735 Generally, a disease or disease state progresses along levels or stages in which the effects of the disease become increasingly severe.

737 The level of disease state may be influenced by the physiological state of the cells in the sample.

[0076]

741 As used herein, the terms "therapy" or "treatment regimen" refer to medical steps taken to alleviate or modify the state of a disease, such as the use of pharmacological, surgical, dietary and/or other techniques intended to reduce or eliminate the disease. Treatment course of effects or symptoms.

744 Treatment regimens may include the administration of one or more medications or prescribed doses of surgery.

746 Therapy is beneficial and reduces disease status in most cases, but in many instances the effects of therapy have undesirable effects or side effects.

748 The effectiveness of therapy is also affected by the physiological state of the host, such as age, gender, genetics, weight, symptoms of other diseases, etc.

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[0077]

753 As used herein, the term "pharmacological state" or "pharmacological status" refers to samples that will and/or have been treated with one or more drugs, surgeries, etc., the drug, surgical procedure, etc. may affect the pharmacological state of one or more nucleic acids in the sample, such as de novo transcription, stabilization and/or destabilization as a result of drug interference.

757 The pharmacological state of a sample relates to changes in biological state before, during, and/or after drug treatment, and can serve as diagnostic or predictive functions as taught herein.

759 Some changes following medical treatment or surgery may be related to the disease state and/or may not be related to side effects of the therapy.

761 Changes in pharmacological status may be a result of the duration of therapy, the type and dose of medications prescribed, compliance with a given course of treatment, and/or the ingestion of unprescribed medications.

[0078]

767 As used herein, the term "biological state" refers to the state of the transcriptome (which is the entire collection of RNA transcripts) of a cell sample isolated and purified for analysis of expression status.

769 The biological state reflects the physiological state of the cells in the sample by measuring the adequacy and/or activity of cellular components, characterization in terms of morphological phenotypes, or a combination of the methods described for detecting transcripts.

[0079]

775 As used herein, the term "expression profile" refers to the relative abundance of RNA, DNA or protein abundance, or activity levels.

777 Expression profiling can be, for example, a measure of transcriptional status or translational status by a variety of methods and using gene chips, gene arrays, beads, multiplex PCR, quantitative PCR, run-on assays, Northern blot analysis, Western blot analysis, protein expression, fluorescence-activated cell sorting (FACS), enzyme-linked immunosorbent assay (ELISA), chemical fluorescence studies, enzymatic assays, proliferation studies, or any other method, equipment, and system, using any of a variety of For determining and/or analyzing gene expression, those used above are readily commercially available.

[0080]

786 As used herein, the term "transcriptional state" of a sample includes the nature and relative abundance of RNA species, particularly mRNA, present in the sample.

788 The entire transcriptional state of a sample, that is, the combination of RNA properties and abundance, is also referred to here as the transcriptome.

790 Generally, a fraction of the total relative composition of the entire collection of RNA species in the sample is measured.

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[0081]

795 As used herein, the term "modular transcription vector" refers to transcriptional expression data that reflects "the proportion of differentially expressed genes."

797 For example, for each module, the proportion of transcripts that are differentially expressed between at least two groups (eg, healthy subjects vs. patients).

799 The vector was derived from a comparison of samples from both groups.

800 The first analysis step is used to select disease-specific transcript sets within each module.

801 Subsequently, there is "expression level".

802 Group comparisons for a given disease provide a list of transcripts that are differentially expressed for each module.

804 Different diseases were found to result in different sets of modular transcripts.

805 It is then possible to calculate vectors for each module (or modules) of a single sample at this expression level by averaging the expression values for a subset of disease-specific genes identified as differentially expressed.

807 This method enables the generation of a map of modular expression vectors for a single sample, such as those described in the modular map disclosed herein.

809 These vector module profiles represent the average expression level of each module (rather than a subset of differentially expressed genes) that can be obtained for each sample.

[0082]

814 Using the present invention, it is possible to identify and differentiate diseases not only at the module level, but also at the gene level; that is, two diseases may have the same vector (same proportion of differently expressed transcripts, same "polarity"), however the genetic makeup of the vector may still be disease-specific.

817 Gene-level expression offers the obvious benefit that the resolution of the analysis is greatly improved.

818 Further, the present invention utilizes combinatorial transcription markers.

819 As used herein, the term "combination of transcriptional markers" refers to the numerical average of the expression of multiple genes (subsets of modules) compared to using a single gene as a marker (and the composition of these markers may be disease-specific of).

822 The combined transcriptional marker approach is unique because users are able to develop multivariate microarray integration to assess disease severity in patients using, for example, SLE, or derive expression vectors disclosed herein.

825 Most importantly, the results obtained using the present invention's combinatorial modules for transcriptional markers have been found to be reproducible between microarray platforms, thereby providing greater reliability for registration approval.

[0083]

831 Gene expression monitoring systems for use in the present invention may include customized gene arrays with a limited and/or basic number of genes that are specific and/or tailored for one or more target diseases.

833 Unlike conventionally used pan-genome arrays, the present invention not only provides the application of these general arrays in retrospective gene and genome analysis (without using a specific platform), but more importantly, it provides the development of customized arrays. , the custom array provides a selectable set of

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genes for analysis without the need for thousands of other unrelated genes.

837 A clear advantage of the optimized arrays and modules of the present invention over existing technologies is the reduction in financial costs (eg cost per analysis, materials, equipment, time, labor, training, etc.) and, more importantly, also Reduces the environmental cost of producing pan-arrays (when most of the data is irrelevant).

841 The module of the invention enables for the first time the design of simple, customized arrays that provide optimized data using a minimum number of probes and maximize the signal-to-noise ratio.

843 By reducing the total number of genes for analysis, it is possible, for example, to reduce the need to prepare thousands of expensive platinum masks used in lithography in the preparation of pan-gene chips that produce A lot of irrelevant data.

846 The invention can be used if the limited probe set (or sets) of the invention are combined with the following methods, or any other methods, devices and systems (easily commercially available) for measuring and/or analyzing gene expression. Used together, it is possible to completely avoid the need for microarrays: for example, digital optical chemical arrays, bead arrays, beads (such as Luminex), multiplex PCR, quantitative PCR, sequential analysis, Northern blot analysis, and even for protein analysis such as Western blot Analysis, 2D and 3D gel protein expression, MALDI, MALDI-TOF, fluorescence-activated cell sorting (FACS) (cell surface or intracellular), enzyme-linked immunosorbent assay (ELISA), chemical fluorescence studies, enzyme analysis, value-added research.

[0084]

857 The "molecular fingerprint system" of the present invention can be used in different cells or tissues, in different subpopulations of the same cells or tissues, in different physiological states of the same cells or tissues, in different developmental stages of the same cells or tissues, or In different cell populations of the same tissue, other diseases and/or normal cell controls can be used to facilitate comparative analysis of expression.

861 In some cases, normal or wild-type expression data may come from samples analyzed at or near the same time, or it may be expression data obtained or selected from an existing gene array expression database, e.g. Public databases, such as NCBI Gene Expression Omnibus database.

[0085]

867 As used herein, the term "differentially expressed" refers to cellular components (eg, nucleic acids, proteins, enzyme activities, etc.) that vary between two or more samples (eg, disease samples and normal samples) measurement value.

870 Cellular components may be on or off (present or absent), up-regulated compared to controls or down-regulated compared to controls.

872 For applications using gene chips or gene arrays, differential gene expression of nucleic acids such as mRNA or other RNA (miRNA, siRNA, hnRNA, rRNA, tRNA, etc.) may be used to distinguish cell types or nucleic acids.

875 Most generally, the transcriptional status of a cell is measured by quantitative reverse transcriptase (RT) and/or quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), genomic expression analysis, post-translational analysis, analysis of genomic DNA Modification, translocation, in situ hybridization, etc. are

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completed.

[0086]

882 For some disease states, cellular or morphological differences can be identified, especially at early levels of the disease state.

884 The present invention avoids the need to identify a specific mutation or gene(s) by looking at the gene module of the cell itself or, more importantly, by looking at the cellular RNA expression of genes from immune effector cells that function in their normal physiology. It acts under certain conditions, that is, during immune activation, immune tolerance or even immune weakness.

888 Although genetic mutations can cause significant changes in the expression levels of a group of genes, biological systems often compensate for the changes by altering the expression of other genes.

890 The result of these intrinsic compensatory responses is that many perturbations may have small effects on the observable phenotype of the system, yet have profound effects on the composition of cellular components.

892 Similarly, the actual copy number of a gene transcript may not increase or decrease, however, the persistence or half-life of the transcript may be affected, resulting in a large increase in protein production.

894 The present invention eliminates the need to detect actual signals by, in one embodiment, observing effector cells (eg, leukocytes, lymphocytes, and/or subpopulations thereof) rather than individual signals and/or mutations.

[0087]

900 The skilled artisan will readily recognize that samples may be obtained from a variety of sources, including, for example, single cells, collections of cells, tissues, cell cultures, and the like.

902 In certain cases it may even be possible to isolate sufficient RNA from cells found, for example, in urine, blood, saliva, tissue or biopsy samples, etc.

904 In certain circumstances, sufficient cells and/or RNA may be obtained from mucosal secretions, feces, tears, plasma, ascites fluid, interstitial fluid, intradural, cerebrospinal fluid, sweat or other body fluids.

906 The source of the nucleic acid, such as from a tissue or cell source, may include a tissue biopsy sample, one or more sorted cell populations, cell cultures, cell clones, transformed cells, biopsy samples, or single cells.

908 The source of the tissue may include, for example, brain, liver, heart, kidney, lung, spleen, retina, bone, neural, lymph nodes, endocrine glands, reproductive organs, blood, nerve, vascular tissue, and olfactory tissue. epithelium.

[0088]

914 The present invention includes the following basic components, which may be used alone or in combination, namely, one or more data mining algorithms; one or more module level analysis processes; characterization of blood leukocyte transcription modules; aggregated module data Applications in multivariate analysis for molecular diagnosis/prediction of human disease; and/or visualization of data and results at the module level.

918 Using the present invention, it is also possible to develop and analyze combined transcriptional markers, which may further be summarized in a single multivariate score.

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[0089]

923 The explosion in data acquisition rates has stimulated the development of mining tools and algorithms for exploiting microarray data and biomedical knowledge.

925 Approaches aimed at uncovering the modular organization and function of transcriptional systems include promising approaches for identifying powerful molecular signatures of disease.

927 In effect, this analysis can transform the understanding of large-scale transcriptional studies by conceptualizing microarray data beyond individual genes or sets of genes.

[0090]

932 The inventors recognize that current microarray-based research is facing significant challenges, with analysis of data being notoriously "noisy", that is, data is difficult to interpret and difficult to compare well between laboratories and platforms.

935 For the analysis of microarray data, a widely accepted approach begins with the identification of subsets of genes that are differentially expressed between study groups.

937 Next, the user then attempts to "make sense" of the resulting gene series using pattern recognition algorithms and existing scientific and technological knowledge.

[0091]

942 The inventors developed a strategy that emphasizes the selection of biologically relevant genes early in the analysis rather than dealing with the large variability between platforms.

944 Briefly, the method involves the identification of transcript components that characterize a given biological system, for which improved data mining algorithms are developed to analyze and extract groups of co-expressed genes or transcripts from large data sets. module.

[0092]

950 Pulmonary tuberculosis (PTB) is a major and increasing cause of morbidity and mortality worldwide caused by Mycobacterium tuberculosis (M. tuberculosis).

952 However, most individuals infected with M. tuberculosis remain asymptomatic, maintaining the infection in a latent form, and it is thought that this latent state is maintained by an active immune response.

954 Blood is the pipeline of the immune system and is therefore an ideal biomaterial from which the health and immune status of an individual can be established.

956 Therefore, using microarray technology to assess the activity of the entire genome in blood cells, we identified distinct and reciprocal blood transcriptional biomarker signatures in patients with active and latent tuberculosis.

959 These labels were also different from those in control individuals.

960 The signature of latent tuberculosis, which shows overexpression of immune cytotoxic genes in whole blood, may be useful in identifying protective immune factors against M. tuberculosis infection, since these patients

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are infected but most do not Overt disease develops.

963 This distinct transcriptional biomarker signature from active and latent TB patients could also be used to diagnose infection and to monitor response to treatment with anti-mycobacterial drugs.

965 Additionally, signatures in active TB patients can help identify factors involved in immunopathogenesis and may lead to strategies for immunotherapeutic intervention.

967 The present invention relates to an earlier application claiming rights to the use of blood transcriptional biomarkers in the diagnosis of infection.

969 However, this prior application did not disclose the existence of biomarkers for active and latent tuberculosis, but focused instead on children with other acute infections (Ramillo, Blood, 2007).

[0093]

974 The identification of transcriptional signatures in blood from latent and active TB patients can be used to test patients suspected of having M. tuberculosis infection, as well as for health screening/early detection of the disease.

977 The present invention also makes it possible to assess the response to treatment with antimycobacterial drugs.

978 In this context, trials may also be particularly valuable in drug testing, especially in the evaluation of drug treatments in patients with multidrug resistance.

980 Further, the present invention may be used to obtain immediate, interval and long-term data from immune signatures of latent tuberculosis to better define protective immune responses in vaccination trials.

982 At the same time, signatures in active TB patients can help identify factors involved in immune pathogenicity and may lead to strategies for immunotherapeutic intervention.

[0094]

987 The blood represents a reservoir and migration zone for cells of the innate and adaptive immune system, including neutrophils, dendritic cells, and monocytes or B and T lymphocytes, respectively, which are exposed during infection. Infectious agents in tissues.

990 Because of this, whole blood from infected individuals provides a viable source of clinically relevant material using previously described methods for intratissue cancer (Alizadeh AA., 2000; Golub, TR., 1999; Bittner, 2000) and autoimmunity (Bennet, 2003; Baechler, EC, 2003; Burczynski, ME, 2005; Chaussabel, D., 2005; Cobb, JP., 2005; Kaizer, EC., 2007; Allantaz, 2005; Allantaz, 2007) and Gene expression microarrays for the study of inflammation (Thach, DC., 2005) and infectious diseases (Ramillo, Blood, 2007) in blood or tissue (Bleharski, JR et al., 2003) can yield unbiased molecular phenotypes.

996 Microarray analysis of gene expression in blood leukocytes identified diagnostic and predictive gene expression signatures, which has led to a better understanding of the mechanisms of disease onset and response to treatment (Bennet, L 2003; Rubins, KH., 2004; Baechler, EC, 2003; Pascual, V., 2005; Allantaz, F., 2007; Allantaz, F., 2007).

1000 These microarray methods have been attempted to study active and latent TB, however so far only a small number of differentially expressed genes have been identified (Jacobsen, M., Kaufmann, SH., 2006; Mistry, R, Lukey, PT, 2007) and in a relatively small number of patients (Mistry, R., 2007), it may not be sufficient to differentiate between other inflammatory and infectious diseases.

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[0095]

1007 To define the immune signature of TB, blood from active and latent TB patients and controls was analyzed; very strict clinical criteria were used to select patients.

1009 Patients were recruited from London, UK, where the number of active TB cases is increasing and, most importantly, the risk of confounding co-infections is minimal, resulting in a robust signature that can differentiate between latent and active TB.

1012 Using microarrays to analyze the entire genome, subsequent data mining revealed that a large number of the genes found were differentially expressed at statistically significant levels in all groups of patients, including active and latent patients, as well as healthy controls. .

1015 Subsequently, a novel approach based on a modular data mining strategy was used, which provides a basis for the selection of clinically relevant transcriptional biomarkers for analysis of blood microarray transcriptional profiles in SLE and other diseases, and Changed our understanding of disease onset (Chaussabel, 2008, Immunity).

1019 The modular map defined in this study provides a way to organize complex data and reduce its dimensionality, while still maintaining the large number of genes expressed in human blood, allowing the observation of specific disease fingerprints (Chaussabel, 2008, Immunity).

1022 Using this modular approach, a well-defined modular transcriptional signature was obtained that was distinct and reciprocal in the whole blood of active and latent TB patients, and also differed from healthy controls.

1024 The biomarkers described here improve the diagnosis of PTB and further help define host factors important in protection against M. tuberculosis in patients with latent TB, as well as those major factors involved in the immunopathogenesis of active TB, and thus used to reduce and control TB disease.

[0096]

1030 Patients, materials and methods

[0097]

1034 Participant recruitment and patient description: Participants were recruited from St. Mary's Hospital TBClinic, Imperial College Healthcare NHS Trust, London, and healthy controls were recruited from volunteers at the National Institute for Medical Research (NIMR), Mill Hill, London.

1037 This study was approved by the NHS Research Ethics Committee at St Mary's Hospital (LREC), London, UK.

1039 All participants (18 years and older) gave written informed consent.

1040 Recruited participants met strict clinical criteria to determine their provisional study group before being assigned to the final analysis group.

1042 Cohorts of patients and controls were as follows: (i) active PTB based on clinical diagnosis and subsequent laboratory isolation of M. tuberculosis confirmed by tuberculosis culture; (ii) latent TB - determined by a positive tuberculin skin test (TST, using 2TU tuberculin (Serum Statens Institute, Copenhagen, Denmark), $\geqslant$6 mm if not vaccinated with BCG; $\geqslant$15 mm if vaccinated with BCG, with a positive result using

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interferon gamma release assay (IGRA, specifically Quantiferon-TBGold In-tube assay, Cellestis, Australia)).

1048 This IGRA assay measures reactivity to an antigen by IFN-γ release from whole blood (ESAT-6/CFP-10/TB 7.7-present in M. tuberculosis, but not present in most environmental tubercle bacilli or M. bovis BCG vaccine).

[0098]

1054 Patients with latent TB must also have evidence of exposure to an infectious TB case, either through close home or work contact, or as a "new attendee" to the ward; or as a person with an incidentally found TST positive who was not exposed to the infection. Patients with signs were not selected for this study.

1057 (iii) Healthy volunteer controls (BCG-vaccinated and unvaccinated, ≤14 mm or ≤5 mm, respectively, by TST; and negative by IGRA).

1059 Patients who were pregnant, known to be immunosuppressed, taking immunosuppressive therapy, or had diabetes or autoimmune disease were also ineligible and excluded from the study.

1061 HIV-positive individuals (only 1% of TB patients in London have previously undiagnosed HIV) were excluded from the study.

1063 Blood from patients with active and latent PTB was collected for this study before administration of any antimycobacterial drugs and subsequently at set intervals as a longitudinal component of the subsequent study.

[0099]

1069 For each participant, expected detailed clinical information was collected and entered into a web-accessible database developed by the inventors.

1071 Using this recorded clinical data, as well as the immune-based analysis described previously, 15 of the 58 participants were excluded from the study because they did not meet the study's standard criteria.

1073 This resulted in the following populations: 6 healthy volunteers who were not vaccinated with BCG; 6 healthy volunteers who were vaccinated with BCG; 17 patients with latent TB; and 14 patients with active PTB. All these samples were used for RNA isolation. .

1076 After processing was carried out, one sample from an active TB patient did not produce sufficient globulin-depleted RNA and was excluded from the final analysis.

[0100]

1081 RNA sampling, extraction, and processing for microarray: Whole blood from the above patient population was collected into Tempus tubes (Applied Biosystems, Foster City, CA, USA) and stored at -20° C to -80° C prior to RNA extraction. ℃.

1084 Total RNA was extracted using the PerfectPure RNA Blood Kit (5PRIME Inc, Gaithersburg, MD, USA).

1085 The samples were homogenized with 100% cold ethanol, vortexed, and then centrifuged at 4000<i>g for 60 min at 0° C. to remove the supernatant.

1087 Add 300 μl of lysis solution to the pellet and vortex.

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Subsequent RNA binding, DNase treatment, washing and RNA elution steps were performed according to the manufacturer's instructions.

Isolated total RNA was globin removed using the GLOBINclear™ 96-well formatting kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions.

Total and globulin-depleted RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA).

After processing was carried out, one sample from an active TB patient did not yield sufficient amounts of globulin-depleted RNA and was therefore excluded from the final analysis.

Biotinylated amplified RNA target (cRNA) was subsequently prepared from the globulin-depleted RNA using the Illumina CustomPrep RNA Amplification Kit (Ambion, Austin, TX, USA).

Labeled cDNA was hybridized overnight on a Sentrix Human-6 V2 BeadChip Array (>48,000 probes, Illumina Inc, San Diego, CA, USA), washed, blocked, stained and plated on an Illumina BeadStation 500 following the manufacturer's protocol. scanning.

Illumina' s BeadStudio version 2 software was used to generate signal intensity values from the scans, subtract background, and compare individual microarrays to the median average intensity across all samples (corrected for each chip).

This corrected data was used for all subsequent data analyses.

[0101]

Microarray data analysis: Gene expression analysis software program, Genespring version 7.1.3 (Agilent) was used to perform statistical analysis and hierarchical clustering of samples.

Differentially expressed genes were selected and clustered as described in the Results and Figures section.

[0102]

Results and discussion

[0103]

Blood tags separate active and latent TB patients from each other and from healthy control individuals: To determine whether blood samples from active and latent TB patients carry active and latent TB compared to healthy controls. A step-by-step analysis was performed based on the gene expression signatures that could be distinguished by comparison.

After filtering out undetected transcripts and genes that deviated less than 2-fold from the median (i.e., had a flat profile), the RNA samples obtained from whole blood RNA samples (from active and latent TB and healthy controls) Pearson correlation coefficient of expression profiles performed unsupervised cluster analysis of 6269 genes (Fig. 1).

The unsupervised analysis identified distinct signatures found to correspond to different clinical phenotypes: in patients with active pulmonary TB (active PTB); and in individuals with latent TB (latent sexual TB).

The grouping of samples was not perfect (10 of 13 patients with active and 11 of 17 patients with latent TB).

However, the majority of active PTB and latent TB patients in this group from the training set of patients were

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shown to have clear and distinct transcriptional signatures.

1131 Importantly, these labels were shown to be present across a wide range of ethnicities selected for this study, including White, Black African, Asian Indian, Asian Bengali, Asian Other, White Irish, White Mixed Race, Black Caribbean (these data The details are not shown.

1134)

[0104]

1138 The list of 6269 genes was further analyzed using non-parametric statistical group comparisons (Kruskal-Wallis test) to identify genes that were differentially expressed between groups.

1140 Using a moderately stringent multiple comparison correction to control for type I error (Benjamini-Hochberg correction), 1473 genes were differentially expressed/expressed between active and latent TB and healthy controls (P<0.01) (Figure 2; and the 1473 genes listed in the long table submitted with this document).

1144 These clustered genes were then linked to relevant findings in the literature.

1145 For the ontological term "immune response", filtering of these genes resulted in a list of 158 such genes (Fig. 3A-D; Table 2).

1147 The expression/phenomenon profile of 158 genes (Figures 3A-3D) enables the differentiation of active TB patients from latent TB patients and from healthy control individuals.

[0105]

1152 Table 2.

1153 List of 158 genes annotated with biological processes of Gene Ontology terms: immune response, and found to be differentially expressed between active TB and other clinical groups (p<0.01)

[0112]

1158 Genes overexpressed/expressed in active TB: Of interest are a large number of IFN-related/inducible genes that are expressed: e.g. interferon (IFN)-inducible genes such as SOCS1, STAT1, PML (TRIM19), TRIM22, various guanylate-binding proteins, and various other IFN-inducible genes noted in Table 2. Interestingly, however, these were not evident in patients with latent TB, although these The representation/expression of IFN-γ transcripts in whole blood is actually higher than in active TB patients.

1163 To focus on this, specific families of genes (some known to be up-regulated by IFN and others not) were further studied, including the TRIM family.

[0113]

1168 A subset of TRIMS are overexpressed/expressed in active TB: the three-domain protein (TRIM) family of proteins is characterized by a discreet structure (Reymond, A., EMBO J., 2001) and has been shown to have diverse functions, Including E3 ubiquitin ligase activity, induction of cell proliferation, differentiation and apoptosis, and immune cell signal transduction (Meroni, G., Bioessays, 2005).

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1172 Their involvement involves protein-protein interactions, autoimmunity, and development (Meroni, G., Bioessays, 2005).

1174 Furthermore, many TRIM proteins were found to have antiviral activity and may be involved in innate immunity (Nisole, F, 2005, Nat.

1176 Rev.

1177 Microbiol.

1178; Gack, MU., 2007, Nature).

1179 Interestingly, 30 TRIM transcripts (some overlapping probes) were shown to be expressed in active TB, and some were also expressed in latent TB and healthy control blood (Fig. 4; Table 3).

1181 Most of these TRIMs were previously shown to be expressed in human and mouse macrophages and dendritic cells (Rajsbaum, 2008, EJI; Martinez, FO., J.Imm., 2006) and to be regulated by IFN, however TRIM (Rajsbaum, 2008, EJI), which was shown to be necessarily expressed in DCs or in T cells, was not detected or found to be differentially expressed in active or latent TB relative to healthy control blood. Express.

1186 Interestingly, TRIM 5, 6, 19 (PML), 21, 22, 25, 68 were found to be overrepresented/expressed; although others were underrepresented/expressed: TRIM 28, 32, 51, 52, 68.

1188 Of interest is a group of TRIMs that are highly expressed in active TB but undetectable in latent TB and healthy controls, and four of them (TRIMs 5, 6, 21, 22) have been shown to concentrate on human chromosome 11 and has been reported to have antiviral activity (Song, B., 2005, J. Virol; Li, X, Virology, 2007).

1192 However, a group of TRIMs were found to be low-expressed in the blood of active TB patients relative to latent TB and healthy controls, including TRIMs 28, 32, 51, 52, 68 and those reported to be either or not derived from human blood. expressed in macrophages (TRIM 51), or expressed only in undifferentiated monocytes (TRIM-28, 52) or unactivated macrophages or alternatively activated macrophages (TRIM-32), or (TRIM-68) is only upregulated to low levels in differentiated activated macrophages from human blood (Martinez, FO., J. Imm., 2006).

[0114]

1201 table 3.

1202 TRIM genes differentially expressed in active pulmonary tuberculosis, latent tuberculosis and healthy controls

[0118]

1207 Selective overexpression/expression of specific immunomodulatory ligands in active TB patients: Analysis of distinct transcriptional profiles shows that transcripts from genes CD274 (PDL1) and PCDLG2 (PDL2, CD273) are exclusively expressed in active TB patients medium expression (Figure 5A and B).

1210 These molecules have previously been shown to be involved in the regulation of immune responses to acute and chronic viral infections (A Sharpe, Ann.

1212 Rev.

1213 Imm.).

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1214 These molecules function as inhibitory costimulatory receptors for the molecule PD1 in T cell and APC interactions, and blockage of this pathway has been shown to restore the proliferation and proliferation of antigen-specific T cells in HIV, hepatitis B, and hepatitis C infections. effect function.

[0119]

1220 Genes with low expression/expression in active TB: Strikingly, multiple genes known to be expressed on T cells (some also expressed on NK and B cells) were found to be extremely low in the blood of active TB patients. Downregulated/underrepresented (Figure 3D), (but not in latent TB or healthy controls, included CD3, CTLA-4, CD28, ZAP-70 (T, NK and B cells), IL-7R, CD2 (Also on B cells), SLAM (also on NK cells), CCR7, GATA-3 (also on NK cells).

1225 This may mean that in T, NK and B cells, gene expression is downregulated during active PTB, or that cells have been re-recruited elsewhere (such as the lung) due to infection with M. tuberculosis.

1227 Blood from different patient groups is now studied using flow cytometry analysis, but also by transcriptional analysis of purified populations of T cells from different patient groups.

[0120]

1232 More rigorous statistical analysis of transcriptional profiles in latent and active patients relative to healthy controls.

1234 Previously, statistical group comparisons were further performed using the nonparametric Kruskal-Wallis test by identifying genes that were differentially expressed between groups. However, in order to control type I errors, the most stringent multiple comparison correction (Bonferroni correction) was now used.

1237 Under this increased stringency, 46 genes ($P < 0.1$) and 18 genes ($P < 0.05$) were identified as differentially expressed between groups (Figs. 6 and 7; Tables 4 and 5).

1239 Among the 46 genes, a large number of IFN-inducible genes such as STAT-1, GBP and IRF-1 were still observed to be overexpressed/expressed in the blood of active TB patients and in latent patients or Downregulated or unchanged in healthy controls.

1242 A significant number of these genes were also found to be overexpressed/expressed in the blood of active TB patients, with genes still extracted even in the most stringent analysis (Bonferroni corrected, <0.05).

1244 Of the panel of 46 genes observed in active TB, only 3 transcripts were downregulated/underrepresented, which included IL-7R (expressed in T cells), chemokine receptor CXCR3 (at higher statistics lost under stringency) and α II-spectrin.

1247 The low expression/expression of CXCR3 is of interest as this chemokine receptor has been shown to be highly expressed on Th1 cells required for protection against mycobacterial infections, which may reflect their Inhibit or migrate out of the bloodstream to reach infected tissue.

1250 Table 5 includes 18 genes where IL7R and SPTAN1 are underrepresented/expressed in active PTB and all others are overrepresented/expressed and diagnostic for active disease.

[0121]

1255 Table 4 Genes differentially expressed between active TB and other clinical groups.

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[0125]

1259 table 5.

1260 Eighteen genes were significantly differentially expressed between active TB and other clinical groups.

[0127]

1264 Improved identification between patients and healthy controls with active and latent TB: The aforementioned methods, although able to identify active TB from latent TB and healthy controls, were less able to identify active TB in all three clinical groups. identification between.

1267 To select identified genes, subsequent methods were used.

1268 First, genes expressed in blood from healthy individuals were compared with latent TB patients using the Wilcoxon-Mann-Whitney test at $p<0.005$, resulting in 89 discriminating genes.

1270 Genes expressed in blood from healthy individuals were then compared with active TB patients again using the Wilcoxon-Mann-Whitney test and the most stringent Bonferroni correlation coefficient at $p < 0.5$, resulting in a set of 30 discriminating Gene.

1273 This list was combined to give a total of 119 identified genes (Table 6).

1274 This list of genes was then used to review the data set for all clinical groups using unsupervised cluster analysis by Pearson's correlation coefficient.

1276 This analysis yielded three distinct clinical group clusters (Figs. 8A to 8F): one cluster consisted of 11 of the 13 active TB patients (Fig. 8, cluster C); the second cluster consisted of 17 Consisting of 16 latent TB patients and 1 active TB patient (Fig. 8, cluster B); the third cluster contained all 12 healthy controls included in this study, plus 1 active TB patient and One latent TB solitary patient (Figure 8, cluster A).

1280 For each of Figures 8A through 8F, the clusters of patients/clinical groups are represented horizontally and the clusters of genes are represented vertically.

1282 The expression/phenomenon profile of all 119 genes (Fig. 8A) is now in a form such that all three clinical groups are identifiable from each other: that is, such that active TB, latent TB and healthy individuals are identifiable from each other, the individual clinical groups show Unique expression/performance profiles of these 119 genes or subsets thereof.

1286 The skilled artisan will recognize that 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 15, 20, 25, 30, 35, or more genes can be placed in a data set representing gene clustering, the genes can be compared individually or together with other clusters among the clusters of clinical groups A (healthy), B (latent), C (active), and each clinical group can be shown to derive from these 119 A unique expression/expression profile of a gene.

[0128]

1293 Specifically, Figure 8B demonstrates the genes ST3GAL6, PAD14, TNFRSF12A, VAMP3, BR13, RGS19, PILRA, NCF1, LOC652616, PLAUR(CD87), SIGLEC5, B3GALT7, IBRDC3(NKLAM), ALOX5AP(FLAP), MMP9, ANPEP(APN), NALP12, CSF2RA, IL6R(CD126), RASGRP4, TNFSF14(CD258), NCF4, HK2, ARID3A, PGLYRP1(PGRP) are lowly expressed/lowly expressed in the blood of latent TB patients, while in healthy individuals or active This was not the case in the blood of TB

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patients.

[0129]

1302 The genes shown in Figure 8C are ABCG1, SREBF1, RBP7(CRBP4), C22orf5, FAM101B, S100P, LOC649377, UBTD1, PSTPIP-1, RENBP, PGM2, SULF2, FAM7A1, HOM-TES-103, NDUFAF1, CES1, CYP27A1, FLJ33641, GPR177, MID1IP1(MIG-12), PSD4, SF3A1, NOV(CCN3), SGK(SGK1), CDK5R1, LOC642035 showed overexpression/overexpression in the blood of healthy control individuals, while in latent TB patients low expression/low expression in the blood and, to a large extent, low expression/low expression in the blood of active TB patients.

[0130]

1311 In Figure 8D, the gene profiles of ARSG, LOC284757, MDM4, CRNKL1, IL8, LOC389541, CD300LB, NIN, PHKG2, and HIP1 are shown to be overexpressed/overrepresented in the blood of healthy individuals, however, in the blood of latent and active TB Low expression/low expression in patient's blood.

1314 In contrast, the genes PSMB8 (LMP7), APOL6, GBP2, GBP5, GBP4, ATF3, GCH1, VAMP5, WARS, LIMK1, NPC2, IL-15, LMTK2, STX11 (FHL4) in Figure 8D were found in the blood of active TB. Shown to be overexpressed/overrepresented, yet underexpressed/underrepresented in latent TB patients as well as healthy control individuals.

[0131]

1321 In Figure 8E, the gene profiles of FLJ11259 (DRAM), JAK2, GSDMDC1 (DF5L) (FKSG10), SIPAIL1, [2680400] (KIAA1632), ACTA2 (ACTSA), and KCNMB1 (SLO-B) were all found in active TB patients. over-expression/over-representation in blood, whereas it was not expressed or even under-expressed/under-represented in blood from latent TB patients and healthy control individuals.

1325 In contrast, genes SPTAN1, KIAAD179(Nnp1)(RRP1), FAM84B(NSE2), SELM, IL27RA, MRPS34, [6940246](IL23A), PRKCA(PKCA), CCDC41, CD52(CDW52), [3890241](ZN404), MCCC1 (MCCA/B), SOX8, SYNJ2, FLJ21127, and FHIT are low expressed/underrepresented in the blood of active TB patients, but not in latent TB patients or healthy control individuals, here They are overexpressed/overexpressed.

[0132]

1333 Many of the genes listed in Figures 8D and 8E (out of the 119 genes selected according to this method as described above) were found to be overexpressed/overrepresented in the blood of active TB patients. These genes are useful in active, latent TB. common in patients as well as healthy controls (genes underlined in Figure 8D and Figure 8E are included in Figure 7, in the gene list in Table 5, 18 genes, $p<0.05$; the genes expressed in italics in Figures 8D and 8E are included in Figure 6, in the gene list in Table 4, 46 genes, $P<0.1$).

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[0133]

1342 Figure 8F shows that the gene profile of CD52 (CD W 52), [3890241] (ZNF404), MCCC1 (MCCA/B), SOX8, SYNJ2, FLJ21127, and FHIT is low expressed/lowly expressed in the blood of active TB patients, however, in This is not the case in the blood of latent TB patients or healthy control individuals, where it is overexpressed/overexpressed.

1346 This is also represented (overlaid) in Figure 8E.

1347 Genes CDKL1(p42), MICALCL, MBNL3, RHD, ST7(RAY1), PPR3R1, [360739](PIP5K2A), AMFR, FLJ22471, CRAT(CAT1), PLA2G4C, ACOT7(ACT)(ACH1), RNF182, KLRC3(NKG2E), HLA-DPB1 is underexpressed/underexpressed in the blood of healthy control individuals, however overexpressed/overexpressed in the blood of latent TB patients, and overexpressed/overexpressed in the blood of most active TB patients (Figure 8F).

1352 In summary, the overall expression profile of all 119 genes in Figure 8A (divided in Figures 8B-8F for ease of understanding of genes and specificity between clinical states) that differentiates the infected (active TB and latent TB) patients were distinguished from uninfected patients (healthy controls) and, in addition, two groups of infected patients were distinguished, namely active and latent TB patients.

1356 By this approach, multiple genes overexpressed in the blood of active TB patients were the same genes identified using the most stringent statistical screen (shown in Figure 7, Table 6), and many were IFN-inducible of and/or involved in cellular traffic by endocytosis and/or lipid metabolism.

[0134]

1362 Table 6.

1363 Genes that were found to be significantly differentially expressed between latent and healthy, or between active and healthy, were distinguished when used in combination using the unsupervised Pearson correction coefficient clustering algorithm (119 genes) Active, healthy and latent.

[0140]

1369 Different and reciprocal immune signatures in active and latent TB were revealed by a modular approach.

1370 In order to obtain further information on pathogenesis, the data from the calibrated individual chips were further analyzed using the stable module analysis framework just described, which is based on predefined clustering of gene transcripts shown in They are commonly expressed in a wide range of diseases and often exhibit molecular or cellular clustering at the functional level (Chaussabel et al., 2008, Immunity).

[0141]

1377 Since the goal of this analysis was to obtain functional information about the genes contained in each set of transcriptional signatures, the analysis focused on a subset of patients that clustered closely together in our previous analysis, excluding outliers. The reason for this is that such groups are more likely to show common pathways and processes involved in the disease process.

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[0142]

Nine patients with active TB, six healthy controls, and nine patients with latent TB were selected and used in the module analysis.

Comparisons were performed separately, such that nine active TB patients were compared with six healthy controls in one analysis, followed by nine latent patients with the same six healthy controls in a separate analysis. Compare.

Transcripts were screened to exclude any not detected in at least two individuals of any group compared.

Statistical comparisons of patients and healthy controls were then performed (non-parametric Wilcoxon-Mann-Whitney test, $P<0.05$) to identify genes differentially expressed between patient groups and healthy controls.

These differentially expressed genes were then divided into those that were up-regulated/over-represented in the disease group compared to controls, and those that were down-regulated/under-represented in the disease group compared to controls.

These lists are then analyzed on modules via a module basis.

Genes differentially expressed in various modules are either significantly overexpressed or significantly underexpressed.

To ensure identification, each module must have >25% of total gene changes in the aspect of expression, and the number of genes changed in a specific aspect must be >10.

To illustrate the overall transcriptional changes, active TB versus healthy controls, or latent TB versus healthy controls, are arranged on a grid, with individual positions corresponding to different modules according to their original definition.

Dot intensity shows the proportion of transcripts that are differentially expressed in the direction shown to the total number of transcripts detected in that module, while dot color shows the polarity of the change (red: overexpression/expression, blue : low expression/performance).

In addition to this, module coordinates can be associated with functional annotations, thus facilitating data interpretation (Chaussabel, Immunity, 2008; and Figures 9 and 10).

[0143]

The modular profile of active TB compared to healthy controls (Figure 9, Tables 7A-P; and Table 8) showed a different profile than the profile of latent TB compared to healthy controls (Figure 10, Table 7A-P; and Table 8). F; and Table 9).

Indeed, these module profiles generated independently from active TB and latent TB showed opposite patterns of gene expression/expression compared to healthy controls in modules showing changes in both disease states.

Genes in module M2.1 associated with cytotoxic cells were low expressed/represented in active TB (36% - Of the genes listed in Table 6F, 18 out of 50 genes examined in the module were low expressed /expressed) and were overexpressed/expressed in latent TB (43% - 22 of the 51 genes listed in Table 7B overexpressed/expressed in the module).

On the other hand, several genes ("inflammation") in M3.2 and M3.3 (genes listed in Tables 6J and 6K) are overexpressed/expressed in patients with active TB, whereas in latent TB Low expression/expression in TB

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patients (genes listed in Tables 7E and 7F).

1425 Similarly, genes in M1.5 ("myeloid lineage") are overexpressed/represented in active TB (genes listed in Table 6D), yet are underexpressed/represented in latent TB (genes listed in Table 7A genes).

1427 Genes in module M2.10 (which do not form a coherent functional module but are composed of apparently distinct sets of genes) are low expressed/represented in latent TB (genes listed in Table 7D), however is not expressed or has low expression/expression in active TB compared to controls.

1430 One of these genes is the toll-like receptor adapter, TRAM, which is downstream of TLR-4 (LPS) and TLR-3 (dsRNA) signaling (Akira, Nat.

1432 Rev.

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[0144]

1437 For Tables 7A to 7O, relative corrected expression for active TB is given as expression in active patients relative to controls.

1439 In Tables 8A and 8F, the relative corrected expression for latent TB is given as expression in healthy controls relative to latent patients.

[0145]

1444 Table 7A M1.2PTBv.

1445 Control, genes overexpressed in active TB.

[0148]

1449 Table 7B M1.3PTBv.

1450 Control, genes with low expression in active TB.

[0152]

1454 Table 7C M1.4PTBv.

1455 Control, genes underrepresented in active TB

[0156]

1459 Table 7D M1.5PTBv.

1460 Control, genes overexpressed in active TB.

[0160]

1464 Table 7E M1.8PTBv.

1465 Control, genes underrepresented in active TB.

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[0165]

1469 Table 7F M2.1PTBv.

1470 Control, genes underrepresented in active TB.

[0169]

1474 Table 7G M2.4PTBv.

1475 Control, genes underrepresented in active TB.

[0173]

1479 Table 7H M2.8PTBv.

1480 Control, genes underrepresented in active TB.

[0179]

1484 Table 7I M3.1PTBv.

1485 Control, genes overexpressed in active TB.

[0186]

1489 Table 7J M3.2PTBv.

1490 Control, genes overexpressed in active TB.

[0191]

1494 Table 7K M3.3PTBv.

1495 Control, genes overexpressed in active TB.

[0196]

1499 Table 7L M3.4PTBv.

1500 Control, genes underrepresented in active TB.

[0204]

1504 Table 7M M3.6PTBv.

1505 Control, genes underrepresented in active TB.

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[0209]

1509 Table 7N M3.7PTBv.

1510 Control, genes underrepresented in active TB.

[0216]

1514 Table 7O M3.8PTBv.

1515 Control, genes underrepresented in active TB.

[0222]

1519 Table 7P M3.9PTBv.

1520 Control, genes underrepresented in active TB.

[0229]

1524 Table 8A M1.5LTBv.

1525 Control, genes underrepresented in latent TB.

[0234]

1529 Table 8B M2.1LTBv.

1530 Control, genes overexpressed in latent TB.

[0238]

1534 Table 8C M2.6LTBv.

1535 Control, genes underrepresented in latent TB.

[0244]

1539 Table 8D M2.10LTBv.

1540 Control, genes underrepresented in latent TB.

[0247]

1544 Table 8E M3.2LTBv.

1545 Control, genes underrepresented in latent TB.

[0254]

1549 Table 8F M3.3LTBv.

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1550 Control, genes underrepresented in latent TB

[0262]

1554 The active TB group showed 5281 genes that were differentially expressed compared to healthy controls, while the latent group showed only 3137 genes that were differentially expressed compared to controls, possibly reflecting Overexpression/expression of genes in the cytotoxic module revealed a more modest (albeit clear) active immune response.

1558 By way of illustration and not limitation of the invention, these results likely explain the observed changes in additional modules in patients with active TB compared with controls, but not in latent TB compared with controls. .

1561 These include expression/expression in M1.2 (platelets, genes listed in Table 7A), and in M1.3 (B cells, genes listed in Table 7B) and M2.8 (T cells, The latter may be expected for genes with low expression/expression (genes listed in Table 7H), as in the T cell response to M. tuberculosis infection, T cells may be recruited at the site of infection and/or in chronic Suppressed during infection.

1565 Genes in module M2.4, downregulated/expressed (genes listed in Table 7G) include transcripts encoding members of the ribosomal protein family whose expression is altered in acute infection and sepsis (Calvano, 2005; Thach, 2005)), and genes in this module have been shown to have low expression in patients with SLE, lung transplants, and in humans infected with Streptococcus(S)pneumoniae (Chaussabel, Immunity, 2005).

1569 In active TB, the largest set of overexpressed genes (60 genes out of 90 tested, Table 7I) was observed in module M3.1 (IFN-inducible), a set of genes that are conserved Consistent with the function of IFN-γ, however genes in this module were not differentially expressed in latent TB patients who had controlled infection compared with controls.

1573 In active TB, genes are lowly expressed in multiple gene-containing modules (M3.4, M3.6, M3.7, M3.8, and M3.9, genes listed in Tables 7L-7P), so The genes do not appear to be coherent functional modules but comprise a distinct collection of genes and have been observed to be low expressed in lung transplant recipients (Chaussabel., 2008, Immunity).

[0263]

1580 Based on transcriptional analysis of whole blood and using this modular profiling approach, patients with active TB can be distinguished from those with latent TB.

1582 Furthermore, in comparing the active TB module maps obtained in this study with other module maps created for different diseases, it is clear that the active TB module maps were significantly lower in patients with SLE, transplantation, melanoma, or S. pneumoniae. of TB patients have distinct global transcriptional profiles (Figure 9) (Chaussabel, 2008, Immunity).

1586 Specific modules such as M2.4, which include transcripts encoding members of the ribosomal protein family and are found in active TB, SLE, lung transplant patients, and humans infected with S. pneumoniae, may be common to a variety of diseases. is low expression.

1589 However, genes in other modules are less extensively affected, such as 3.1 (IFN-inducible), which despite being overexpressed in active TB (Figure 9) and SLE (Chaussabel, 2008, Immunity), This is not the case in other diseases, especially in S.pneumoniae, which does not show differential gene expression in M3.1

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compared with controls.

1593 The transcriptional profile in SLE differs from that in active TB with respect to overexpression or underexpression of genes in multiple other modules.

1595 Similarly, although overexpression of genes in modules M3.2 and M3.3 ("inflammation"), M1.2 (platelets) and M1.5 ("myeloid") was observed in active TB and S.pneumoniae, as well as low expression of genes in modules M3.4, 5, 6, 7, 8 and 9 (non-functional consensus modules), these diseases can still be differentiated by this method due to comparison with controls, genes in modules M2.2 (neutrophils), M2.3 (erythrocytes), M3.5 (non-functionally identical modules) are overexpressed in S.pneumoniae but not differentially affected in TB.

1601 Therefore, by retaining the complexity and importance of the data, yet organizing the complex data and reducing its dimensionality, it is possible to differentiate different infectious and inflammatory diseases through the transcriptional profile of the blood (Chaussabel, 2008, Immunity).

[0264]

1607 We identified a discreet set of differential and reciprocal transcriptional signatures in the blood of latent and active TB patients.

1609 Specifically, active TB patients showed overexpression/representation of genes in functional IFN-inducible, inflammatory and myeloid modules, which on the other hand were downregulated/underrepresented in latent TB.

1612 Active TB patients show increased/overexpression of the immune regulatory genes PDL-1 and PDL-2, which may contribute to immunopathogenesis in TB.

1614 Blood from latent TB patients shows overexpression/expression of genes in the cytotoxic module that may contribute to the protective response to M. tuberculosis infection in these patients and may inform experimental vaccination in clinical trials The effect of providing biomarkers.

1617 We believe the success of our preliminary studies relies on our adoption of rigorous clinical guidelines, concomitant immunoreactivity studies conducted to support the attribution of potential factors, improved quality of RNA collection and isolation, and advanced high-throughput whole-genome microarray platforms., and sophisticated data mining tools that preserve the size of gene expression in a readable format (Chaussabel et al.).

1622 This finding is valuable as a diagnostic tool for latent and active TB and may lead to an understanding of the mechanisms underlying immune protection (latent TB) versus immune pathogenesis (active TB) (these transcriptional differences). implicit), and the design of novel therapies for protection or immunotherapy in active TB to achieve faster therapeutic effects with antimycobacterial drugs.

[0265]

1629 It is contemplated that any embodiment discussed in this specification with respect to any method, kit, reagent or composition of the invention can be implemented, and vice versa.

1631 Further, the compositions of the invention may be used to carry out the methods of the invention.

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[0266]

1635 It is to be understood that the specific embodiments described herein are provided by way of illustration and not as limitations of the invention.

1637 The principal features of the invention may be employed in different embodiments without departing from the scope of the invention.

1639 Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, various equivalents to the specific procedures described herein.

1641 Such equivalents are considered to be within the scope of the invention and covered by the claims.

[0267]

1645 All publications and patent applications mentioned in this specification are indicative of the level of skill of a person skilled in the art to which the invention relates.

1647 All publications and patent applications 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.

[0268]

1653 The use of the word "a" or "an", when used with the term "comprising" in the claims and/or description, may mean "a", however it may also mean "a" or "an" "Plural", "at least one" and "one or more than one" have the same meaning.

1656 Although the specification supports the definition of "and/or" only referring to alternatives, the term "or" is used in the claims to mean "and/or" unless it is explicitly stated that it only refers to alternatives or optional The options are mutually exclusive.

1659 In this application, the term "about" is used to indicate that a numerical value includes variations that are inherent to equipment errors, the method used to determine the numerical value, or variations that exist among the subjects under study.

[0269]

1665 As used in this specification and the claims (or claims), the word "comprising" (and any form of including such as "comprise" and "comprises"), "having having)" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include)") or "containing" (and any form of containing such as "contains" and "contain") is inclusive or open-bounded and does not exclude additional, unlisted elements Or method steps.

[0270]

1673 The term "or combinations thereof" as used herein refers to all permutations and combinations of the individuals listed before that term.

1675 For example, "A, B, B, or combinations thereof" is expected to include at least one of A, B, C, AB, AC, BC, or

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ABC, and if order matters in a particular context, also BA, CA, CB, CBA, BCA, ACB, BAC or CAB.

1677 Continuing with the example, repeated combinations of one or more elements or terms are also explicitly included, such as BB, AAA, MB, BBC, AAABCCCC, CBBAAA, CABABB, etc.

1679 Skilled artisans understand that there is typically no numerical limitation on the use of items or terms in any combination unless otherwise expressly stated in the text.

[0271]

1684 All compositions and/or methods disclosed and claimed herein may be prepared and performed in accordance with the present disclosure without undue experimentation.

1686 Although the compositions and methods of the invention have been described in terms of preferred embodiments, it will be apparent to those skilled in the art that variations may be made in the compositions and/or methods described herein, as well as in the steps of the methods. Or the order of the steps may be changed without departing from the principle, spirit and scope of the invention.

1690 All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and principle of the invention as defined by the appended claims.

[0272]

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Espacenet

CN102150043A Blood transcriptional signature of mycobacterium tuberculosis infection

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Blood transcriptional signature of mycobacterium tuberculosis infection

Abstract

The present invention includes methods, systems and kits for distinguishing between active and latent mycobacterium tuberculosis infection in a patient suspected of being infected with mycobacterium tuberculosis, and distinguishing such patients from uninfected individuals, the method including the steps of obtaining a gene expression dataset from a whole blood obtained sample from the patient and determining the differential expression of one or more transcriptional gene expression modules that distinguish between infected and non-infected patients, wherein the dataset demonstrates an aggregate change in the levels of polynucleotides in the one or more transcriptional gene expression modules as compared to matched non- infected patients, thereby distinguishing between active and latent mycobacterium tuberculosis infection.

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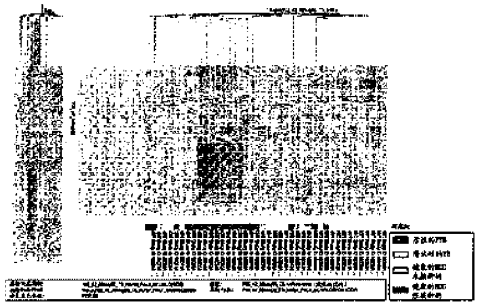
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(54)发明名称

结核分枝杆菌感染的血液转录标签

(57)摘要

本发明包括在疑似结核分枝杆菌感染的患者中，用于区分活性和潜伏性的结核分枝杆菌感染，以及将该患者从未感染的个体区分出来的方法、系统和试剂盒，所述方法包括以下步骤：从得自患者的样品的全血获得基因表达数据集，确定区分感染的和未感染的患者的一个或多个转录基因表达模块的有差别的表达，其中所述数据集显示了与相对应的未感染的患者相比，在一个或者多个转录基因模块中的多核苷酸水平的总体变化，从而区分活性的和潜伏性的结核分枝杆菌感染。



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1. 一种用于在疑似结核分枝杆菌感染的患者中区分活性的和潜伏性的结核分枝杆菌感染的方法，所述方法包括：

从来自所述患者的全血样品中获得基因表达数据集；

测定一个或者多个转录基因表达模块的有差别的表达，所述模块区分被感染的患者和未感染的个体，其中所述数据集显示了与相对应的未感染的个体相比，在所述一个或者多个转录基因表达模块中多核苷酸水平的总体变化；以及

根据所述在活性的和潜伏性的感染之间有差别的一个或者多个转录基因表达模块，区分活性的和潜伏性的结核分枝杆菌（TB）感染。

2. 权利要求 1 所述的方法，其进一步包括使用所述测定的对比基因产物信息来制定诊断的步骤。

3. 权利要求 1 所述的方法，其进一步包括使用所述测定的对比基因产物信息来制定预测的步骤。

4. 权利要求 1 所述的方法，其进一步包括使用所述测定的对比基因产物信息来制定治疗计划的步骤。

5. 权利要求 1 所述的方法，其进一步包括区分具有潜伏性的 TB 的患者和活性的 TB 患者的步骤。

6. 权利要求 1 所述的方法，其中所述模块包括在模块 M1.2、M1.3、M1.4、M1.5、M1.8、M2.1、M2.4、M2.8、M3.1、M3.2、M3.3、M3.4、M3.6、M3.7、M3.8 或者 M3.9 中的基因的数据集，从而检测活性的肺部感染。

7. 权利要求 1 所述的方法，其中所述模块包括在模块 M1.5、M2.1、M2.6、M2.10、M3.2 或者 M3.3 中的基因的数据集，从而检测潜伏性的感染。

8. 权利要求 1 所述的方法，其中下列基因在活性的肺部感染中被下调：CD3、CTLA-4、CD28、ZAP-70、IL-7R、CD2、SLAM、CCR7 和 GATA-3。

9. 权利要求 1 所述的方法，其中图 9 的表达谱指示活性的肺部感染。

10. 权利要求 1 所述的方法，其中图 10 的表达谱指示潜伏性的感染。

11. 权利要求 1 所述的方法，其中在模块 M3.4、M3.6、M3.7、M3.8 和 M3.9 中基因的低表达指示活性的感染。

12. 权利要求 1 所述的方法，其中在模块 M3.1 中的基因的过表达指示活性的感染。

13. 权利要求 1 所述的方法，其进一步包括通过测定模块 M2.2、M2.3 和 M3.5 中的基因表达，区分 TB 感染与其他细菌感染的步骤，所述模块由分枝杆菌之外的感染的外周血液单核细胞或者全血过表达。

14. 权利要求 1 所述的方法，其进一步包括在潜伏性的和活性的 TB 患者血液中区分有差别的和相互的转录标签的步骤，其使用下列模块的两个或更多个：对于活性的肺部感染，为 M1.3、M1.4、M1.5、M1.8、M2.1、M2.4、M2.8、M3.1、M3.2、M3.3、M3.4、M3.6、M3.7、M3.8 或者 M3.9，以及对于潜伏性的感染，为模块 M1.5、M2.1、M2.6、M2.10、M3.2 或者 M3.3。

15. 权利要求 1 所述的方法，其中相对于健康的患者，在活性的肺部 TB 感染中上调的基因选自表 7A、7D、7I、7J 和 7K。

16. 权利要求 1 所述的方法，其中相对于健康的患者，在活性的肺部 TB 感染中下调的基因选自表 7B、7C、7E、7F、7G、7H、7L、7M、7N、7O 和 7P。

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17. 权利要求 1 所述的方法，其中相对于健康的患者，在潜伏性的 TB 感染中上调的基因选自表 8B。

18. 权利要求 1 所述的方法，其中相对于健康的患者，在潜伏性的 TB 感染中下调的基因选自表 8A、8C、8D、8E 和 8F。

19. 一种用于在疑似结核分枝杆菌感染的患者中区分活性的和潜伏性的结核分枝杆菌感染的方法，所述方法包括：

从具有活性的结核分枝杆菌感染的第一个临床组得到第一基因表达数据集，从具有潜伏性的结核分枝杆菌感染患者的第二个临床组得到第二基因表达数据集，和从未感染个体的临床组得到的第三基因表达数据集；

产生基因簇数据集，所述数据集包括第一、第二和第三数据集的任意两者之间基因有差别的表达；以及

测定表达 / 表现的独特的图谱，所述图谱指示潜伏性的感染、活性的感染或者健康。

20. 权利要求 19 所述的方法，其中将各个临床组分成表 6 中 119 个基因中的各个的独特的表达 / 表现图谱。

21. 权利要求 19 所述的方法，其中比较第一和第三数据集的数值，并且从中减去来自第三数据集的数据集的数值。

22. 权利要求 19 所述的方法，其中比较第二及第三数据集的数值，并且从中减去来自第三数据集的数据集的数值。

23. 权利要求 19 所述的方法，其进一步包括比较两个不同的数据集的数值，并且减去剩下的数据集的数值的步骤，从而区分具有潜伏性的感染的患者、具有活性的感染的患者以及未感染的个体。

24. 权利要求 19 所述的方法，其进一步包括使用所述测定的对比基因产物信息来制定诊断或者预测的步骤。

25. 权利要求 19 所述的方法，其进一步包括使用所述测定的对比基因产物信息来制定治疗计划的步骤。

26. 权利要求 19 所述的方法，其进一步包括区分具有潜伏性的 TB 的患者和活性的 TB 患者的步骤。

27. 权利要求 19 所述的方法，其进一步包括测定下述基因的表达水平：ST3GAL6、PAD14、TNFRSF12A、VAMP3、BRI3、RGS19、PILRA、NCF1、LOC652616、PLAUR(CD87)、SIGLEC5、B3GALT7、IBRDC3(NKLAM)、ALOX5AP(FLAP)、MMP9、ANPEP(APN)、NALP12、CSF2RA、IL6R(CD126)、RASGRP4、TNFSF14(CD258)、NCF4、HK2、ARID3A、PGLYRP1(PGRP)，其在潜伏性的 TB 患者的血液中低表达 / 低表现，而在健康的个体或者活性的 TB 患者的血液中不是如此。

28. 权利要求 19 所述的方法，其进一步包括测定下述基因的表达水平：ABCG1、SREBF1、RBP7(CRBP4)、C22orf5、FAM101B、S100P、LOC649377、UBTD1、PSTPIP-1、RENBP、PGM2、SULF2、FAM7A1、HOM-TES-103、NDUFAF1、CES1、CYP27A1、FLJ33641、GPR177、MID1IP1(MIG-12)、PSD4、SF3A1、NOV(CCN3)、SGK(SGK1)、CDK5R1、LOC642035，其在健康的对照个体的血液中过表达 / 过表现，而在潜伏性的 TB 患者的血液中低表达 / 低表现，并在活性的 TB 患者的血液中低表达 / 低表现。

29. 权利要求 19 所述的方法，其进一步包括测定下述基因的表达水平：ARSG、

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LOC284757、MDM4、CRNKL1、IL8、LOC389541、CD300LB、NIN、PHKG2、HIP1，其在健康的个体的血液中过表达 / 过表现，在潜伏性和活性的 TB 患者的血液中低表达 / 低表现

30. 权利要求 19 所述的方法，其进一步包括测定下述基因的表达水平：PSMB8(LMP7)、APOL6、GBP2、GBP5、GBP4、ATF3、GCH1、VAMP5、WARS、LIMK1、NPC2、IL-15、LMTK2、STX11(FHL4)，其在活性的 TB 的血液中过表达 / 过表现，并在潜伏性的 TB 患者和健康的对照个体的血液中低表达 / 低表现。

31. 权利要求 19 所述的方法，其进一步包括测定下述基因的表达水平：FLJ11259(DRAM)、JAK2、GSDMDC1(DF5L)(FKSG10)、SIPA1L1、[2680400](KIAA1632)、ACTA2(ACTSA)、KCNMB1(SLO-BETA)，其在来自活性的 TB 患者的血液中过表达 / 过表现，并在来自潜伏性的 TB 患者和健康的对照个体的血液中低表达 / 低表现。

32. 权利要求 19 所述的方法，其进一步包括测定下述基因的表达水平：SPTAN1、KIAAD179(Xnp1)(RRP1)、FAM84B(NSE2)、SELM、IL27RA、MRPS34、[6940246](IL23A)、PRKCA(PKCA)、CCDC41、CD52(CDW52)、[3890241](ZN404)、MCCC1(MCCA/B)、SOX8、SYNJ2、FLJ21127、FHIT，其在活性的 TB 患者的血液中低表达 / 低表现，而在潜伏性的 TB 患者或者健康的对照个体的血液中不是如此。

33. 权利要求 19 所述的方法，其进一步包括测定下述基因的表达水平：CDKL1(p42)、MICALCL、MBNL3、RHD、ST7(RAY1)、PPR3R1、[360739](PIP5K2A)、AMFR、FLJ22471、CRAT(CAT1)、PLA2G4C、ACOT7(ACT)(ACH1)、RNF182、KLRC3(NKG2E)、HLA-DPB1，其在健康的对照个体的血液中低表达 / 低表现，在潜伏性的 TB 患者的血液中过表达 / 过表现，并且在活性的 TB 患者的血液中过表达 / 过表现。

34. 一种用于在疑似结核分枝杆菌感染的患者中区分活性的和潜伏性的结核分枝杆菌感染的方法，所述方法包括：

从全血样品中获得基因表达数据集；

将所述基因表达数据集分类到一个或多个转录基因表达模块中；以及

将区分活性的和潜伏的结核分枝杆菌感染的一个或者多个转录基因表达模块的有差别的表达进行绘图，从而区分活性的和潜伏性的结核分枝杆菌感染。

35. 权利要求 34 所述的方法，其中所述数据集包括 TRIM 基因。

36. 权利要求 34 所述的方法，其中所述数据集包括 TRIM 基因，并且 TRIM 5、6、19(PML)、21、22、25、68 在活性的肺部 TB 中过表现 / 表达。

37. 权利要求 34 所述的方法，其中所述数据集包括 TRIM 基因，并且 TRIM 28、32、51、52、68 在活性的肺部 TB 中低表现 / 表达。

38. 一种在疑似结核分枝杆菌感染的患者中诊断具有活性的和潜伏性的结核分枝杆菌感染的患者的方法，所述方法包括检测得自全血的一个或者多个转录基因表达模块的有差别的表达，所述模块区分感染的和未感染的患者，其中全血显示了与相对应的未感染的患者相比，在所述一个或多个转录基因表达模块中多核苷酸水平的总体变化，从而区分活性的和潜伏性的结核分枝杆菌感染。

39. 权利要求 38 所述的方法，其进一步包括使用所述测定的对比基因产物信息来制定诊断的步骤。

40. 权利要求 38 所述的方法，其进一步包括使用所述测定的对比基因产物信息来制定

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预测的步骤。

41. 权利要求 38 所述的方法，其进一步包括使用所述测定的对比基因产物信息来制定治疗计划的步骤。

42. 权利要求 38 所述的方法，其中所述模块包括在模块 M1.2、M1.3、M1.4、M1.5、M1.8、M2.1、M2.4、M2.8、M3.1、M3.2、M3.3、M3.4、M3.6、M3.7、M3.8 或者 M3.9 中的基因的数据集，从而检测活性的肺部感染。

43. 权利要求 38 所述的方法，其中所述模块包括在模块 M1.5、M2.1、M2.6、M2.10、M3.2 或者 M3.3 中的基因的数据集，从而检测潜伏性的感染。

44. 权利要求 38 所述的方法，其中以下基因在活性的肺部感染中被下调：CD3、CTLA-4、CD28、ZAP-70、IL-7R、CD2、SLAM、CCR7 和 GATA-3。

45. 权利要求 38 所述的方法，其中图 9 的模块的表达谱为活性的肺部感染的诊断。

46. 权利要求 38 所述的方法，其中图 10 的模块的表达谱为潜伏性的感染的诊断。

47. 权利要求 38 所述的方法，其中在模块 M3.4、M3.6、M3.7、M3.8 和 M3.9 中的基因的低表达指示活性的感染。

48. 权利要求 38 所述的方法，其中在模块 M3.1 中的基因的过表达指示活性的感染。

49. 权利要求 38 所述的方法，其进一步包括通过测定模块 M2.2、M2.3 和 M3.5 中的基因表达区分 TB 感染和其他的细菌感染的步骤，所述模块在分枝杆菌之外的感染中，由外周血液单核细胞或者全血过表达。

50. 权利要求 38 所述的方法，其进一步包括在潜伏性的和活性的 TB 患者的血液中使用两个或更多个以下模块区分不同的和相互的转录标签的步骤：对于活性的肺部感染，为 M1.3、M1.4、M1.5、M1.8、M2.1、M2.4、M2.8、M3.1、M3.2、M3.3、M3.4、M3.6、M3.7、M3.8 或者 M3.9，以及对于潜伏性的感染，为模块 M1.5、M2.1、M2.6、M2.10、M3.2 或者 M3.3。

51. 一种用于在疑似结核分枝杆菌感染的患者中诊断具有活性和潜伏性的结核分枝杆菌感染的患者的试剂盒，所述试剂盒包含：

用于从所述患者获得基因表达数据集的基因表达检测器；以及

能够比较所述基因表达与得自全血的预设的基因模块数据集的处理器，所述数据集区分感染的和未感染的患者，其中全血显示了与相对应的未感染的患者相比，在一个或者多个转录基因表达模块中的多核苷酸水平的总体变化，从而区分活性的和潜伏性的结核分枝杆菌感染。

52. 一种诊断具有活性的和潜伏性的结核分枝杆菌感染的患者的系统，所述系统包含：

来自所述患者的基因表达数据集；以及

能够比较所述基因表达与得自全血的预设的基因模块数据集的处理器，所述数据集区分感染的和未感染的患者，其中全血显示了与相对应的未感染的患者相比，在一个或者多个转录基因表达模块中的多核苷酸水平的总体变化，从而区分活性的和潜伏性的结核分枝杆菌感染，其中对于活性的肺部感染，所述模块选自 M1.3、M1.4、M1.5、M1.8、M2.1、M2.4、M2.8、M3.1、M3.2、M3.3、M3.4、M3.6、M3.7、M3.8 或者 M3.9，以及对于潜伏性的感染，选自模块 M1.5、M2.1、M2.6、M2.10、M3.2 或者 M3.3。

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结核分枝杆菌感染的血液转录标签

[0001] 发明技术领域

[0002] 本发明总的来说涉及结核分枝杆菌感染的领域，更具体地，涉及用于在治疗之前、期间和之后，诊断、预测和监测潜伏性的和活性的结核分枝杆菌感染和疾病进展。

[0003] 长表格

[0004] 本专利申请包括长表格部分。所述表格的拷贝以电子的形式可得自 USPTO 网站 (http://seqdata.uspto.gov/)。该表格的电子拷贝经要求及缴纳 37CFR 1.19(b)(3) 所规定的费用可从 USPTO 获得。

[0005] 发明背景

[0006] 不限制本发明的范围下，将其背景结合结核分枝杆菌感染的鉴定和治疗进行描述。

[0007] 肺结核 (PTB) 是全世界结核分枝杆菌 (M.tuberculosis) 引起的发病和死亡的主要的和增长的起因。然而，多数感染了 M.tuberculosis 的个体保持无症状，将感染保持在潜伏性的形式，并认为该潜伏性的状态是通过主动免疫响应维持的 (WHO ;Kaufmann, SH & McMichael, AJ., Nat Med, 2005)。这受到报道的支持，所述报道显示使用抗 -TNF 抗体治疗患克罗恩病 (Crohn' sDisease) 或者类风湿关节炎 (Rheumatoid Arthritis) 的患者，引起自身免疫症状的改善，然而另一个方面在事先接触 M.tuberculosis(Keane) 的患者中引起 TB 的再活化。对 M.tuberculosis 的免疫响应是多因子的，并包括遗传学上确定的宿主因子，如 Th1 轴上的 TNF 和 IFN-γ 以及 IL-12 (综述于 Casanova, Ann Rev ;Newport)。然而，来自成年肺 TB 患者的免疫细胞能产生 IFN-γ、IL-12 和 TNF，以及 IFN-γ 疗法不帮助改进疾病 (综述于 Reljic, 2007, JInterferon & Cyt Res., 27, 353-63)，说明了更广泛数量的宿主免疫因子牵涉到抗 M.tuberculosis 的保护以及潜伏性的保持中。因此，在潜伏性的或者活性的 TB 中所诱导的宿主因子的认识能提供关于免疫响应的信息，所述免疫响应能够控制 M.tuberculosis 的感染。

[0008] PTB 的诊断可能由于多种原因而困难及有问题。首先，通过显微检查 (涂布阳性) 来证明典型的结核分枝杆菌在痰中的存在仅有 50-70％的灵敏性，并且阳性诊断要求 M.tuberculosis 通过培养进行分离，这可能长达 8 周。除此之外，一些患者的痰是涂布阴性的，或者不能产生痰，故需要通过支气管镜检查法这种介入性操作进行额外的取样。由于 PTB 的诊断中的这些限制，有时涂布阴性的患者要测试结核菌素 (PPD) 皮肤反应 (曼托试验)。然而，结核菌素 (PPD) 皮肤反应不能区分 BCG 接种、潜在或者活性的 TB。针对这个问题，已经开发了证明对特异 M.tuberculosis 抗原的免疫反应性的检测，所述抗原不存在于 BCG 中。然而，对这些 M.tuberculosis 抗原的反应性 (由在 γ 干扰素释放检测 (IGRA) 中通过血细胞产生 IFN-γ 而测量) 并不将潜伏性的与活性的疾病区分开。在临床上，当患者经皮下注射 PPD，在没有临床症状或迹象或者放射学显示的活性的疾病而具有 IGRA 阳性结果时，通过延迟类型的高度灵敏反应来定义潜伏性的 TB。潜伏性的 / 潜在的肺结核 (TB) 的再活化表现出严重的健康危害，具有向其他个体传播的风险，因此反映潜伏性的和活性的 TB 患者的区别的生物标记物在疾病控制中可能是有用的，尤其是由于抗分枝杆菌的药物治

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疗是困难的，并可能导致严重的副作用。

[0009] 发明概述

[0010] 本发明包括用于鉴别潜伏性的与活性的结核（TB）患者的方法和试剂盒（与健康的对照相比）。在一个实施方案中，采用不同的和相互的免疫标签（signature）的血液微阵列分析来确定、诊断、跟踪及治疗潜伏性的与活性的结核（TB）患者。

[0011] 在一个实施方案中，本发明包括在怀疑感染了结核分枝杆菌的患者中，区分活性和潜伏性的结核分枝杆菌感染的方法、系统和试剂盒，所述方法包括下述步骤：从患者的全血样品中获得基因表达数据集；测定一个或多个转录基因表达模块的有差别的表达，所述模块区别感染的患者及未感染的个体，其中所述数据集显示了与所对应的未感染的个体相比，在一个或多个转录基因表达模块中多核苷酸水平的总体变化，并且基于区分活性和潜伏性的感染的一个或者多个转录基因表达模块，区分活性的和潜伏性的结核分枝杆菌（TB）感染。在一个方面，本发明还可以包括使用所测定的对比基因产物信息来制定诊断的步骤。

[0012] 在另一个方面，本方法还可以包括使用所测定的对比基因产物信息来规划预测的步骤，或者使用所测定的对比基因产物信息来制定治疗计划的步骤。在一个可选的方面，本发明可以包括区分具有潜伏的TB的患者与活性的TB患者的步骤。在一个方面，所述模块可以包括模块M1.2、M1.3、M1.4、M1.5、M1.8、M2.1、M2.4、M2.8、M3.1、M3.2、M3.3、M3.4、M3.6、M3.7、M3.8或者M3.9中的基因的数据集，从而检测活性的肺部感染。在另一个方面，所述模块可以包括模块M1.5、M2.1、M2.6、M2.10、M3.2或者M3.3中基因的数据集，从而检测潜伏性的感染。在再另一个方面，下列基因在活性的肺部感染中被下调：CD3、CTLA-4、CD28、ZAP-70、IL-7R、CD2、SLAM、CCR7和GATA-3。在一个具体的方面，图9的模块表达谱指示活性的肺部感染，图10的模块表达谱指示潜伏性的感染。已经发现了在模块M3.4、M3.6、M3.7、M3.8和M3.9中基因的低表达指示活性的感染。也已经发现了在模块M3.1中的基因的过表达指示活性的感染。

[0013] 在本发明的再另一个方面，所述方法还可以包括区分TB感染与其他细菌感染的步骤，其通过测定模块M2.2、M2.3和M3.5中的基因表达进行，这些模块在分枝杆菌之外的感染中，由外周血液单核细胞或者全血过表达。可选地，所述方法可以包括区分在潜伏性的和活性的TB患者血液中有差别的和相互的转录标签的步骤，其使用下列的模块的两个或更多个：M1.3、M1.4、M1.5、M1.8、M2.1、M2.4、M2.8、M3.1、M3.2、M3.3、M3.4、M3.6、M3.7、M3.8或者M3.9（用于活性的肺部感染）以及模块M1.5、M2.1、M2.6、M2.10、M3.2或者M3.3（用于潜伏性的感染）。与健康的患者相比，在活性的肺部TB感染中上调的基因的实例选自表7A、7D、7I、7J和7K。与健康的患者相比，在活性的肺部TB感染中下调的基因的进一步实例选自表7B、7C、7E、7F、7G、7H、7L、7M、7N、7O和7P。在一个具体的方面，相对于健康的患者，在潜伏性的TB感染中上调的基因可以选自表8B。在另一个具体的方面，相对于健康的患者，在潜伏性的TB感染中下调的基因可以选自表8A、8C、8D、8E和8F。

[0014] 本发明的另一个实施方案是在怀疑感染了结核分枝杆菌的患者中区分活性的和潜伏性的结核分枝杆菌感染的方法，所述方法包括下述步骤：从具有活性的结核分枝杆菌感染的第一个临床组得到第一基因表达数据集，从具有潜伏性的结核分枝杆菌感染患者的临床组得到的第二基因表达数据集以及从未感染个体的临床组得到的第三基因表达谱；产生基因簇数据集，所述数据集包含第一、第二和第三数据集的任意两者之间基因有差别的

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表达；并确定表达 / 表现的独特的样式，所述样式指示潜伏性的感染、活性的感染或者健康。在一个方面，将各个临床组按照表 6 中 119 个基因中的每一个分到独特的表达 / 表现图谱中。在另一个方面，第一和第二数据集的数值进行比较，并将来自第二数据集的数据集的数值从中减去。在另一个具体的方面，将第二及第三数据集的数值进行比较，并将来自第三数据集的数据集的数值从中减去。在一个具体的实施方案中，所述方法可以进一步包括比较两个不同的数据集的数值，并将剩下的数据集的数值减去的步骤，从而区分具有潜伏性感染的患者、具有活性的感染的患者以及未感染的个体。在一个方面，本发明可以进一步包括使用所测定的对比基因产物信息来制定诊断或者预测的步骤。在再另一个方面，所述方法包括使用所测定的对比基因产物信息来制定治疗计划的步骤。该方法还可以包括通过分析在基因和患者簇中的表达 / 表现，区分具有潜伏性的 TB 的患者与活性的 TB 患者的步骤。

[0015] 在一个具体的方面，该方法可以进一步包括测定下述基因的表达水平的步骤：ST3GAL6、PAD14、TNFRSF12A、VAMP3、BR13、RGS19、PILRA、NCF1、LOC652616、PLAUR(CD87)、SIGLEC5、B3GALT7、IBRDC3(NKLAM)、ALOX5AP(FLAP)、MMP9、ANPEP(APN)、NALP12、CSF2RA、IL6R(CD126)、RASGRP4、TNFSF14(CD258)、NCF4、HK2、ARID3A、PGLYRP1(PGRP)，其在潜伏性的 TB 患者低表达 / 低表现，而在健康的个体或者活性的 TB 患者的血液中不是如此。在另一个具体的方面，该方法可以进一步地包括测定所述基因的表达水平的步骤：ABCG1、SREBF1、RBP7(CRBP4)、C22orf5、FAM101B、S100P、LOC649377、UBTD1、PSTPIP-1、RENBP、PGM2、SULF2、FAM7A1、HOM-TES-103、NDUFAF1、CES1、CYP27A1、FLJ33641、GPR177、MID1IP1(MIG-12)、PSD4、SF3A1、NOV(CCN3)、SGK(SGK1)、CDK5R1、LOC642035，其在健康的对照个体的血液中过表达 / 过表现，但是在潜伏性的 TB 患者的血液中低表达 / 低表现，并在活性的 TB 患者的血液中低表达 / 低表现。在另一个具体的方面，该方法可以进一步包括测定下述基因的表达水平的步骤：ARSG、LOC284757、MDM4、CRNKL1、IL8、LOC389541、CD300LB、NIN、PHKG2、HIP1，其在健康个体的血液中过表达 / 过表现，在潜伏性和活性的 TB 患者中低表达 / 低表现。在一个具体的方面，该方法可以进一步包括测定下述基因的表达水平的步骤：PSMB8(LMP7)、APOL6、GBP2、GBP5、GBP4、ATF3、GCH1、VAMP5、WARS、LIMK1、NPC2、IL-15、LMTK2、STX11(FHL4)，其在活性的 TB 的血液中过表达 / 过表现，并在潜伏性的 TB 患者和健康的对照个体的血液中低表达 / 低表现。在一个具体的方面，该方法可以进一步包括测定下述基因的表达水平的步骤：FLJ11259(DRAM)、JAK2、GSDMDC1(DFNL)(FKSG10)、SIPA1L1、[2680400](KIAA1632)、ACTA2(ACTSA)、KCNMB1(SLO-BETA)，其在来自活性的 TB 患者的血液中过表达 / 过表现，并在来自潜伏性的 TB 患者和健康的对照个体的血液中低表达 / 低表现。在一个具体的方面，该方法可以进一步包括测定下述基因的表达水平的步骤：SPTAN1、KIAAD179(Nnp1)(RRP1)、FAM84B(NSE2)、SELM、IL27RA、MRPS34、[6940246](IL23A)、PRKCA(PKCA)、CCDC41、CD52(CDW52)、[3890241](ZN404)、MCCC1(MCCA/B)、SOX8、SYNJ2、FLJ21127、FHIT，其在活性的 TB 患者的血液中低表达 / 低表现，而在潜伏性的 TB 患者或者健康的对照个体的血液中不如此。在一个具体的方面，该方法可以进一步包括测定下述基因的表达水平的步骤：CDKL1(p42)、MICALCL、MBNL3、RHD、ST7(RAY1)、PPR3R1、[360739](PIP5K2A)、AMFR、FLJ22471、CRAT(CAT1)、PLA2G4C、ACOT7(ACT)(ACH1)、RNF182、KLRC3(NKG2E)、HLA-DPB1，其在健康对照个体的血液中低表达 / 低表现，在潜伏性的 TB 患者的血液中过表达 / 过表现，

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以及在活性的 TB 患者的血液中过表达 / 过表现。

[0016] 本发明的再另一个实施方案是用于在疑似结核分枝杆菌感染的患者中区分活性的和潜伏性的结核分枝杆菌感染的方法，所述方法包括下述步骤：从全血样品中获得基因表达数据集；将所述基因表达数据集分类到一个或多个转录基因表达模块中；并将区分活性的和潜在的结核分枝杆菌感染的所述一个或者多个转录基因表达模块的有差别的表达进行绘图，从而区分活性的和潜伏性的结核分枝杆菌感染。在一个方面，所述数据集包括 TRIM 基因。在一个方面，所述数据集包括 TRIM 基因，具体地，TRIM5、6、19(PML)、21、22、25、68，在活性的肺部 TB 中过表达 / 表达。在一个方面，TRIM 基因的数据集，包括 TRIM 28、32、51、52、68，在活性的肺部 TB 中低表达 / 表达。

[0017] 本发明的另一个实施方案是在怀疑感染了结核分枝杆菌的患者中诊断具有活性的和潜伏性的结核分枝杆菌感染的方法，所述方法包括：检测得自全血的一个或者多个转录基因表达模块的有差别的表达，所述模块区分感染的和未感染的患者，其中全血证实了与相对应的未感染的患者相比，在所述一个或多个转录基因表达模块中多核苷酸水平的总体变化，从而区分活性的和潜伏性的结核分枝杆菌感染。在另一个方面，所述方法包括下述的一个或多个的步骤：使用所测定的对比基因产物信息制定诊断，使用所测定的对比基因产物信息制定诊断的步骤和使用所测定的对比基因产物信息制定治疗计划的步骤。在一个可选的方面，所述方法可以包括区分潜伏性的 TB 与活性 TB 患者的步骤。在一个方面，所述模块可以包括在下述模块中的基因数据集：M1.2、M1.3、M1.4、M1.5、M1.8、M2.1、M2.4、M2.8、M3.1、M3.2、M3.3、M3.4、M3.6、M3.7、M3.8 或者 M3.9，从而检测活性的肺部感染。在另一个方面，所述模块可以包括在下述模块中的基因数据集：M1.5、M2.1、M2.6、M2.10、M3.2 或者 M3.3，从而检测潜伏性的感染。在再另一个方面，下述基因在活性的肺部感染中被下调，CD3、CTLA-4、CD28、ZAP-70、IL-7R、CD2、SLAM、CCR7 和 GATA-3。在一个具体的方面，在图 9 中的模块的表达谱指示活性的肺部感染，在图 10 中的模块的表达谱指示潜伏性的感染。已经发现了在模块 M3.4、M3.6、M3.7、M3.8 和 M3.9 中的基因的低表达指示活性的感染。还发现了在模块 M3.1 中的基因的过表达指示活性的感染。

[0018] 在本发明的再另一个方面，该方法还包括通过测定模块 M2.2、M2.3 和 M3.5 中的基因表达，区分 TB 感染与其他的细菌感染的步骤，所述模块在分枝杆菌之外的感染中，由外周血液单核细胞或者全血过表达。可选地，该方法可以包括在潜伏性的和活性的 TB 患者的血液中，使用两个或更多个下述模块区分不同的和相互的转录标签的步骤：对于活性的肺部感染，为 M1.3、M1.4、M1.5、M1.8、M2.1、M2.4、M2.8、M3.1、M3.2、M3.3、M3.4、M3.6、M3.7、M3.8 或者 M3.9，对于潜伏性的感染，为模块 M1.5、M2.1、M2.6、M2.10、M3.2 或者 M3.3。相对于健康的患者，在活性的肺部 TB 感染中上调的基因的实例选自表 7A、7D、7I、7J 和 7K。相对于健康的患者，在活性的肺部 TB 感染中下调的基因的其它实例选自表 7B、7C、7E、7F、7G、7H、7L、7M、7N、7O 和 7P。在一个具体的方面，相对于健康的患者，在潜伏性的 TB 感染中上调的基因的实例选自表 8B。在另一个具体的方面，相对于健康的患者，在潜伏性的 TB 感染中下调的基因的实例选自表 8A、8C、8D、8E 和 8F。

[0019] 本发明的另一个实施方案是试剂盒，所述试剂盒用于在怀疑感染了结核分枝杆菌的患者中，诊断具有活性和潜伏性的结核分枝杆菌感染，该试剂盒包括用于从患者获得基因表达数据集的基因表达检测器；以及能够将得自全血的基因表达与预设的基因模块数据

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集进行比较的处理器，所述数据集区分感染的和未感染的患者，其中全血证实了与相对应的未感染的患者相比，在一个或者多个转录基因表达模块中的多核苷酸水平的总体变化，从而区分活性的和潜伏性的结核分枝杆菌感染。

[0020] 再另一个实施方案包括诊断具有活性的和潜伏性的结核分枝杆菌感染的患者的系统，其包括：来自所述患者的基因表达数据集；以及能够将得自全血的基因表达与预设的基因模块数据集进行比较的处理器，所述数据集区分感染的和未感染的患者，其中全血证实了与相对应的未感染的患者相比，在一个或者多个转录基因表达模块中的多核苷酸水平的总体变化，从而区分活性的和潜伏性的结核分枝杆菌感染，其中对于活性的肺部感染而言，所述模块选自 M1.3、M1.4、M1.5、M1.8、M2.1、M2.4、M2.8、M3.1、M3.2、M3.3、M3.4、M3.6、M3.7、M3.8 或者 M3.9，对于潜伏性的感染而言，选自模块 M1.5、M2.1、M2.6、M2.10、M3.2 或者 M3.3。

[0021] 附图的简要说明

[0022] 为了更完全地理解本发明的特征和优点，在此参考本发明的详述以及所附的图，以及其中：

[0023] 图 1 显示了来自 42 个参与者的基因阵列表达，存在于至少 2 个样品中的基因 (PLA2)，与中间数相比上调或者下调 2 倍的基因，通过皮尔逊相关系数集中，来比较活性的 PTB、潜伏性的 TB、健康的 BCG 未接种的对照及健康的 BCG 经接种的对照；

[0024] 图 2 显示了基因阵列表达结果，所述结果得自 PAL2，上调或者下调表达 2 倍的，在各临床组之间筛选在表达中统计上显著的差别，所述筛选使用非参数检验 (Kruskal-Wallis)，$P < 0.01$，以及使用 Benjamini-Hochberg 相关系数 (1473 个基因)，并使用皮尔逊相关系数独立地合并，来比较活性的 PTB、潜伏性的 TB 和健康的对照；

[0025] 图 3A-3D 显示了基因阵列表达结果，其来自 PAL2，2 倍表达上调或者下调的，在各个临床组之间筛选在表达中统计上显著的差别，所述筛选使用非参数检验 (Kruskal-Wallis)，$P < 0.01$，以及使用 Benjamini-Hochberg 矫正，并随后筛选对于基因注释中的生物过程“免疫响应”的基因本体论术语的存在，并使用皮尔逊相关系数独立地集中 (158 个基因)。这 158 个基因分别显示在 4 个图中以便阅览 (图 3A-3D)。

[0026] 图 3A 显示了比较活性的 PTB、潜伏性的 TB、健康 BCG 的未接种的对照及健康 BCG 的经接种的对照的基因阵列表达结果；

[0027] 图 3B 显示了比较活性的 PTB、潜伏性的 TB、健康 BCG 的未接种的对照及健康 BCG 的经接种的对照的基因阵列表达结果；

[0028] 图 3C 显示了比较活性的 PTB、潜伏性的 TB、健康 BCG 的未接种的对照及健康 BCG 的经接种的对照的基因阵列表达结果；

[0029] 图 3D 显示了比较活性的 PTB、潜伏性的 TB、健康 BCG 的未接种的对照及健康 BCG 的经接种的对照的基因阵列表达结果；

[0030] 图 4 显示了来自 42 名参与者的基因阵列表达结果，存在于至少 2 个样品中的基因 (PAL2)，与中位数相比高或者低 2 倍的基因，通过皮尔逊相关系数集中的选择作为 TRIM 的基因，比较了活性的 PTB、潜伏性的 TB、健康 BCG 的未接种的对照及健康 BCG 的经接种的对照；

[0031] 图 5A 显示了来自 42 名参与者的基因阵列表达结果的细节，存在于至少 2 个样品

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中的基因（PAL2），与中位数相比高或者低 2 倍的基因，通过皮尔逊相关系数集中，比较了活性的 PTB、潜伏性的 TB、健康 BCG 的未接种的对照及健康 BCG 的经接种的对照，显示了抑制性免疫调控配体（PDL1/CD274，PDL2/CD273）在活性的 TB 患者中过表达。

[0032] 图 5B 显示了未经筛选的基因阵列表达结果，其证实了 PDL1 仅在活性的 TB 患者中表达。

[0033] 图 6 显示了在基因阵列表达结果中筛选至少存在于 2 个样品中的基因，其与中位数相比上调或下调 2 倍，在组之间表达统计上显著差异（$P < 0.1$，带有 Bonferroni 相关系数的 Kruskal-Wallis 非因数检验）（46 个基因），其中独立地使用皮尔逊相关系数集中，比较了活性的 PTB、潜伏性的 TB、健康 BCG 的未接种的对照及健康 BCG 的经接种的对照；

[0034] 图 7 显示了在基因阵列表达结果中筛选存在于至少 2 个样品中的基因，其与中位数相比“表现得”上调或者下调 2 倍，在组之间表达统计上显著差异（$P < 0.05$，带有 Bonferroni 相关系数的 Kruskal-Wallis 非因数检验）（18 个基因），其中独立地使用皮尔逊相关系数集中，比较了活性的 PTB、潜伏性的 TB、健康 BCG 的未接种的对照及健康 BCG 的经接种的对照；

[0035] 图 8A 显示了将不同统计筛选合并区分全部 3 个临床组的结果，所述筛选应用到筛选存在于至少 2 个样品中的基因列表，其与中位数相比“表现得”上调或者下调 2 倍。在潜伏性的和健康的之间，所示的转录物统计上显著不同地表达（$P < 0.005$，无相关系数的 Wilcoxon-Mann-Whitney 非参数检验），和在活性的和健康的之间，转录物统计上显著不同地表达（$P < 0.5$，具有 Bonferroni 相关系数的 Wilcoxon-Mann-Whitney 非参数检验）- 使用皮尔逊相关系数独立地集中的总计 119 个基因（患者 / 临床组的群组表示于水平方向，基因的群组表示于竖直方向）；这些 119 个基因分别在 5 个进一步的图中显示（图 8B-8F），以便阅览，并显示这些基因的亚组也可能用于区分不同的临床组（也即活性的、潜伏性的和健康的）。

[0036] 图 8B 显示了将基因阵列表达结果筛选至少存在于 2 个样品中的基因，其与中位数相比“表现得”上调或下调 2 倍，转录物在潜伏性的和健康的表达中统计上显著差异（$P < 0.005$，无相关系数的 Wilcoxon-Mann-Whitney 非因数检验），并且在活性的和健康的之间，转录物统计上显著不同地表达（$P < 0.5$，具有 Bonferroni 相关系数的 Wilcoxon-Mann-Whitney 非参数检验）（患者 / 临床组的群组在水平方向上表示，基因的群组在竖直方向上表示）；

[0037] 图 8C 显示了将基因阵列表达结果筛选至少存在于 2 个样品中的基因，其与中位数相比“表现得”上调或下调 2 倍，转录物在潜伏性的和健康的表达中统计上显著差异（$P < 0.005$，无相关系数的 Wilcoxon-Mann-Whitney 非因数检验），并且在活性的和健康的之间，转录物统计上显著不同地表达（$P < 0.5$，具有 Bonferroni 相关系数的 Wilcoxon-Mann-Whitney 非参数检验）；

[0038] 图 8D 显示了将基因阵列表达结果筛选至少存在于 2 个样品中的基因，其与中位数相比“表现得”上调或下调 2 倍，转录物在潜伏性的和健康的表达中统计上显著差异（$P < 0.005$，无相关系数的 Wilcoxon-Mann-Whitney 非因数检验），并且在活性的和健康的之间，转录物统计上显著不同地表达（$P < 0.5$，具有 Bonferroni 相关系数的 Wilcoxon-Mann-Whitney 非参数检验）患者 / 临床组的群组在水平方向上表示，基因的群组

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在竖直方向上表示）；

[0039] 图 8E 显示了将基因阵列表达结果筛选至少存在于 2 个样品中的基因，其与中位数相比“表现得”上调或下调 2 倍，转录物在潜伏性的和健康的表达中统计上显著差异（$P < 0.005$，无相关系数的 Wilcoxon-Mann-Whitney 非因数检验），并且，在活性的和健康的之间，转录物统计上显著不同地表达（$P < 0.5$，具有 Bonferroni 相关系数的 Wilcoxon-Mann-Whitney 非参数检验）（患者 / 临床组的群组在水平方向上表示，基因的群组在竖直方向上表示）；

[0040] 图 8F 显示了将基因阵列表达结果筛选至少存在于 2 个样品中的基因，其与中位数相比上调或下调 2 倍，转录物在潜伏性的和健康的表达中统计上显著差异（$P < 0.005$，无相关系数的 Wilcoxon-Mann-Whitney 非因数检验），并且在活性的和健康的之间，转录物统计上显著不同地表达（$P < 0.5$，具有 Bonferroni 相关系数的 Wilcoxon-Mann-Whitney 非参数检验）（患者 / 临床组的群组在水平方向上表示，基因的群组在竖直方向上表示）；

[0041] 图 9 显示了来自 PTB(9) 与对照 (6) 的基因模块分析的基因阵列表达结果：来自 5281 个基因，筛选 PAL2，通过 Wilcoxon-Mann-Whitney 检验，$p < 0.05$ 和无多次检验校准下，活性的 PTB 和健康的对照在统计上显著不同地表达；以及

[0042] 图 10 显示了来自 PTB(9) 与对照 (6) 的基因模块分析的基因阵列表达结果：来自 3137 个基因，筛选 PAL2，通过 Wilcoxon-Mann-Whitney 检验，$p < 0.05$ 和无多次检验校准下，活性的 PTB 和健康的对照在统计上显著不同地表达。

[0043] 发明详述

[0044] 尽管在下文详细地讨论了本发明不同实施方案的制备和应用，应该理解本发明提供了多种可实施的发明概念，所述概念能够在多种具体的环境下体现。在此讨论的具体实施方案仅仅为了说明产生和应用本发明的具体方式，而不限制本发明的范围。

[0045] 为了便于理解本发明，以下定义了多个术语。在此定义的术语具有本发明相关领域的普通技术人员通常所理解的含义。术语如“一个 (a/an)”、“一种 (a/an)”和“该”不意图仅指单数的实体，而包括一般的类，该类中具体的实例可能使用作为说明。本文使用的该术语用于描述本发明的具体实施方案，然而其使用不限制本发明，除非在权利要求中指出。除非另外限定，本文使用的所有技术及科学术语具有本发明所属领域的技术人员所一般地理解的含义。随后的参考文献为技术人员提供了在本发明中所使用的多个术语的一般定义：Singleton 等人，Dictionary of Microbiology and MolecularBiology(2d ed. 1994)；The Cambridge Dictionary of Science and Technology(Walker ed., 1988)；The Glossary of Genetics, 5TH ED., R. Rieger 等人 (eds.), Springer Verlag(1991)；以及 Hale & Marham, The Harper Collins Dictionary ofBiology(1991)。

[0046] 在本领域中不同的生物化学和分子生物学方法是公知的。例如，分离和纯化核酸的方法详细描述于 WO 97/10365；WO 97/27317；LaboratoryTechniques in Biochemistry and Molecular Biology；Hybridization with NucleicAcid Probes, Part I. Theory and Nucleic Acid Preparation, (P. Tijssen, ed.) Elsevier, N.Y. (1993) 的第一章；Sambrook 等人，Molecular Cloning；ALaboratory Manual, Cold Spring Harbor Press, N.Y., (1989)；以及 CurrentProtocols in Molecular Biology, (Ausubel, F.M 等人, eds.) John Wiley & Sons, Inc., New York(1987-1999)，包括附录。

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[0047] 生物信息学定义

[0048] 如本文所使用的，“对象”指任意目标项目或者信息（通常是文本的，包括名词、动词、形容词、副词、短语、句子、符号、数字符号等等）。因此，对象是能够构成关系的任何事物，以及能够获得、鉴定和/或从源中搜索的任何事物。“对象”包括但不限于目标实体，如基因，蛋白质，疾病，显型，机理，药物等等。在一些方面中，如下文进一步所描述地，对象可能为数据。

[0049] 如本文所使用的，“关系”指在同一单元（如短语、句子、文本的两行或更多行、段落、网页的部分、页面、杂志、论文、书籍等等）中共同出现的对象。其可能为文本、符号、数字及其组合。

[0050] 如本文所使用的，“元数据内容”指根据数据源中的文本组织的信息。元数据可能包括标准的元数据（如都柏林核心元数据）或者可能是标本特异的。元数据格式的实例包括但不限于，用于图书馆目录的机器可读目录（MARC）记录，源描述格式（RDF）以及可扩展标记语言（XML）。元对象可能人工产生或者通过自动化的信息提取运算法则产生。

[0051] 如本文所使用的，“引擎”指实施其他程序的核心或关键功能的程序。例如引擎可以为操作系统或者应用程序中的中心程序，其调节其他程序的全部操作。术语“引擎”还可能指包括可以改变的运算法则的程序。例如可以设计知识发掘引擎，从而其确定关系的方式可能变化，从而反映确定和排序关系的新规则。

[0052] 如本文所使用的，“语义分析”指表示相似概念的词之间的关系的确定，如通过去除后缀或者添加部分或者通过采用同义词词典。“统计分析”指基于计算各项目（词、词根、词干、元语法、短语等等）发生数量的技术。在不限定对象的集合中，用于不同的上下文的相同短语可能代表不同的概念。对短语共同出现的统计分析可能帮助分析语义含糊。“语法分析（Syntacticanalysis）”可以用于通过词类分析进一步地减少模糊。如本文使用的，更一般地将一种或多种该分析称作“语法分析（lexical analysis）”。“人工智能（AI）”指某些方法，通过该方法，非人的设备如计算机实施人认为值得注意的或者“智能的”任务。实例包括鉴定图片，理解口语词汇或者书写的文字，以及解决问题。

[0053] 术语如“数据”、“数据集”及“信息”通常可交换地与使用，“信息”和“知识”也是如此。如本文所使用的，“数据”是最基础的单元，其为依据试验的测量或测量的集合。收集数据以构成信息，但是它基本上独立于此，且可以组合成数据集，即数据的集合。与此相对，信息得自目标，如数据（单元）可能在种族、性别、身高、体重及饮食方面收集，用于发现与心血管疾病风险有关的变量的目的。然而，相同的数据可能用于开发食谱或者产生关于饮食偏好的“信息”，所谓饮食偏好即这样的可能性，在超级市场中特定的产品具有更高的销售可能性。

[0054] 如本文所使用的，术语“数据库”是原始的或者收集的数据的储存库，甚至在数据领域中能找到不同的信息方面。数据库可能包括一个或多个数据集。一般地将数据库进行组织从而能访问、管理并更新（例如，该数据库是动态的）其内容。术语“数据库”及“源”也能在本发明中交换地使用，这是由于数据和信息的原始来源是数据库。然而“源数据库”或者“源数据”一般地指数据，例如，输入到系统中用于鉴定对象及确定关系的无结构的文本和/或有结构的数据。源数据库可能是或者可能不是关系数据库。然而，系统数据库通常包括关系数据库或者某种等效类型的数据库，其储存对象之间的关系的相关数值。

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[0055] 如本文所使用的，“系统数据库”及“关系数据库”可替换地使用，并指数据的一个或多个集合，所述集合组织为包括数据的一套表格，其中的数据组合于预先设定的种类。例如，数据库表可能包括列（例如，属性）所设定的一个或多个种类，而数据库的行可能包括对于列所设定的种类而言独特的对象。因此，对象如基因的性质可能具有其存在、缺失、和/或该基因表达水平的列。关系数据库的一行可能也指“一个集合”，并通常地通过其各列的数值定义。关系数据库的情况中的“域”是一系列有效的数值，例如列可能包括的领域。

[0056] 如本文所使用的，“知识域”指研究的领域，在该领域中系统是可操作的，例如，所有的生物医学数据。应该指出从几个域中合并数据是有益的，例如，生物医学数据及工程数据，这是由于不同的数据有时候能将对于只熟悉一个领域或者探索/研究（一个域）的平常人无法放在一起的事物进行联系。“分布的数据库”指可能在网络上不同点中分布或者复制的数据库。

[0057] 如本文所使用的，“信息”指数据集合，其可能包括数字、字母、数字的集合、字母的集合或由数据的集合导致或者得到的结论。“数据”则为测量或者统计量，并且为信息的基本单元。“信息”可能还包括其他类型的数据，如词语、符号、文字，如无结构的自由的文字、编码等等。“知识”被松散地定义为信息的集合，其提供对于系统的充分的理解，从而为原因和效果进行建模。为了延伸前述的实例，对于人口特征、性别和此前的购买的信息可以用于发展用于食物销售的区域市场战略，而关于国籍的信息可能由购买者用作产品进口的指导方针。重要的是，需指出在数据、信息和知识之间没有严格的界限；这三种术语有时认为是等价的。一般地，数据来自测试，信息来自关联，而知识来自建模。

[0058] 如本文所使用的，“程序”或者“计算机程序”一般地指句法单元，其符合特定的编程语言的规则并包括声明文件和陈述或者说明，可分成“代码段”，其被需要用于解决或者执行特定的功能、任务以及问题。编程语言一般地是用于表达程序的人工语言。

[0059] 如本文所使用的，“系统”或者“计算机系统”一般地指一台或多台计算机，外部设备以及进行数据处理的软件。“用户”或者“系统操作者”一般地包括通过“用户设备”（如计算机，无线设备等），为了数据处理和信息交换而使用计算机网络的人。“计算机”一般地是能够进行实质上的计算的功能性单元，其包括无人为干涉下的多种算术运算和逻辑操作。

[0060] 如本文所使用的，“应用软件”或者“应用程序”一般地指对于应用问题的解决特异性的软件或者程序。“应用问题”一般地是由终端用户提交，其解决需要信息处理。

[0061] 如本文所使用的，“自然语言”指其规则基于现有的使用而不特别地规定的语言，如英语、西班牙语或者中文。如在此所使用的，“人工语言”指其规则在其使用前明确地建立的语言，如计算机编程语言，诸如 C、C++、JAVA、BASIC、FORTRAN 或者 COBOL。

[0062] 如本文所使用的，“统计关联性”指使用一种或者多种排序方案（O/E 比率、强度等等），其中如果比随机几率预期其发生显著地更加频繁，则确定关系是统计相关的。

[0063] 如本文所使用的，术语“共同调控的基因”或者“转录模块”可交换地使用，指特定基因的成组的基因表达谱（如与特定的基因序列相关的信号数值）。各个转录模块在两部分关键的数据之间建立联系，即文献检索部分和得自基因微阵列的实际试验的基因表达数值数据。选入转录模块的基因集合基于对基因表达数据的分析（前述的模块选取运算法则）。示教了此外的步骤的有 Chaussabel, D. & Sher, A. Mining microarray expression data by literatureprofiling. Genome Biol 3, RESEARCH0055(2002), (http://

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genomebiology.com/2002/3/10/research/0055)(相关的部分在此结合作为参考)以及得自感兴趣的疾病或者症状的表达数据,所述疾病或者症状如系统性红斑狼疮、关节炎,淋巴瘤,癌,黑色素瘤,急性感染,自身免疫性疾病,自身炎症性疾病等。

[0064] 下表中列出了用于得到文献检索部分或者对转录模块有贡献的关键词的实例。技术人员应意识到在其他情况下能容易地选择其他术语,如具体的癌症、具体的感染性疾病、移植等等。例如,与T细胞活化有关的那些基因的基因和信号以"M 2.8"的模块ID描述于下文,其中特定的关键词(如淋巴瘤、T-细胞、CD4、CD8、TCR、胸腺、淋巴、IL2)用于确定关键的T-细胞相关的基因,如T-细胞表面标记物(CD5、CD6、CD7、CD26、CD28、CD96);由淋巴谱系细胞表达的分子(淋巴毒素β、IL2诱导的T细胞激酶、TCF7;以及T细胞分化蛋白mal、GATA3、STAT5B)。随后,整个模块通过将来自患者群体的这些基因的数据建立联系(不管平台、存在/缺失和/或上调或者下调)从而产生转录模块。在一些情况下,基因表达谱并不匹配(在此时)这些疾病症状的任何具体的基因簇和数据,然而在"未确定的"模块中发现了特定的生理通路(例如cAMP信号转导、锌指蛋白、细胞表面标记物等等)。实际上,基因表达数据集合可能用于在与关键词检索匹配之前,提取具有协调的表达的基因,也即任意数据集合可能在与第三个数据集合交叉引用之前相互关联。

[0065] 表1.转录模块

[0066]

实例模块I.D.	实例关键词选择	基因表达谱的评估
M 1.1	Ig, 免疫球蛋白, 骨, 骨髓, PreB, IgM, Mu.	血浆细胞:包括编码免疫球蛋白链的基因(*e.g.* IGHM, IGJ, IGLL1, IGKC, IGHD)以及血浆细胞标记物CD38。
M 1.2	血小板, 粘合, 聚合, 内皮的, 血管的	血小板:包括编码血小板糖蛋白的基因(ITGA2B, ITGB3, GP6, GP1A/B),和得自血小板的免疫介质如PPPB(脯氨酸-血小板基

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实例模块 I.D.	实例关键词选择	基因表达谱的评估
		本蛋白)和 PF4 (血小板因子 4)。
M 1.3	免疫受体, BCR, B-细胞, IgG	B-细胞:包括编码 B-细胞表面标记物的基因 (CD72, CD79A/B, CD19, CD22)和其他 B-细胞关联的分子: 早期 B-细胞因子 (EBF), B-细胞连接物(BLNK)和 B 淋巴酪氨酸激酶 (BLK)。
M 1.4	复制, 抑制, 修复, CREB, 淋巴的, TNF-α	未确定。该系列包括 cAMP 信号转导通路的调节物和靶点(JUND, ATF4, CREM, PDE4, NR4A2, VIL2),以及 TNF-α 介导的 NF-KB 活化的抑制物 (CYLD, ASK, TNFAIP3)。
M 1.5	单核细胞, 树突的, MHC, 共同刺激, TLR4, MYD88	骨髓谱系:包括骨髓谱系细胞表达的分子(CD86、CD163、FCGR2A), 其一些涉及病原体识别(CD14、TLR2、MYD88)。本集合还包括 TNF 家族成员(TNFR2、BAFF) 。
M 1.6	锌, 指, P53, RAS	未确定。本集合包括编码信号转导分子的基因, 如活化的 STAT(PIAS1 和 PIAS2)的含有锌指的抑制剂, 或者活化的 T-细胞 NFATC3 的核因子。
M 1.7	核糖体, 翻译的, 40S, 60S, HLA	MHC/核糖体蛋白: 几乎专一地由编码 MHC 1 型分子(HLA-A,B,C,G,E) + β 2-微球蛋白 (B2M) 或者核糖体蛋白 (RPLs、RPSs).的基因形成。
M 1.8	代谢, 生物合成, 复制, 螺旋酶	未确定。包括编码代谢酶的基因(GLS、NSF1、NAT1)及涉及 DNA 复制的因子(PURA、TERF2、EIF2S1)。
M 2.1	NK, 致命物, 细胞溶解的, CD8, Cell 介导的, T-cell, CTL, IFN-g	细胞毒性细胞: 包括细胞毒性的 T-细胞和 NK-细胞表面标记物 (CD8A、CD2、CD160、NKG7、KLRs), 细胞溶解分子(颗粒酶、穿孔蛋白质、粒溶素), 趋化因子 (CCL5, XCL1) 和 CTL/NK-细胞相关分子(CTSW)。
M 2.2	粒细胞, 中性粒细胞, 防御, 骨髓,	中性粒细胞:该集合包括在中性粒细胞颗粒中发现的固有的分子(乳铁转递蛋白: LTF、

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实例模块 I.D.	实例关键词选择	基因表达谱的评估
	髓	抵御素: DEAF1、增加细菌通透性蛋白: BPI, Cathelicidin 抗微生物蛋白: CAMP)。
M 2.3	红细胞, 红, 贫血症,球蛋白, 血红素	红细胞: 包括血红素基因 (HGB) 和其他的红细胞相关的基因(红细胞 alkirin:ANK1, 血型糖蛋白 C: GYPC, 羟甲基胆色烷合成酶: HMBS, 红细胞相关因子: ERAF)。
M 2.4	核蛋白, 60S, 核仁, 组合体, 延长	核糖体蛋白: 包括编码核糖体蛋白的基因 (RPL, RPS), 真核翻译延长因子家族成员 (EEF) 和核仁蛋白 (NPM1 、 NOAL2 、 NAP1L1)。
M 2.5	腺瘤, 间质的, 间叶细胞、树突的、马达	未确定。该模块包括编码免疫相关的(CD40、CD80、CXCL12、IFNA5、IL4R) 以及细胞骨架相关的分子 (肌凝蛋白、浆细胞移动的 Dedicator、多配体蛋白聚糖 2、Plexin C1、Distrobrevin) 的基因。
M 2.6	粒细胞, 单核细胞, 骨髓的, ERK, 坏死	骨髓谱系: 与 M 1.5 相关。包括在骨髓谱系细胞中表达的基因 (IGTB2/CD18, 淋巴毒素 β 受体, 骨髓相关蛋白质 8/14 甲酸基肽受体 1), 如单核细胞和中性粒细胞:
M 2.7	未提取关键词	未确定。该模块主要地包括没有已知功能的转录物。仅有 20 个基因与文献相关, 包括趋化因子样因子超家族的一个成员 (CKLFSF8)。
M 2.8	淋巴瘤, T 细胞, CD4, CD8, TCR, 胸腺, 淋巴的, IL2	T-细胞: 包括 T 细胞表面标记物 (CD5、CD6、CD7、CD26、CD28、CD96) 和由淋巴谱系细胞表达的分子 (淋巴毒素 β、IL2 可诱导的 T 细胞激酶、TCF7、T 细胞分化蛋白 mal、GATA3、STAT5B)。
M 2.9	ERK, 反式激活, 细胞骨架, MAPK, JNK	未确定。包括编码细胞骨架相关分子的基因 (肌动蛋白相关蛋白 2/3、MAPK1、MAP3K1、RAB5A)。还存在的有 T 细胞表达的基因 (FAS、ITGA4/CD49D、ZNF1A1)。
M 2.10	骨髓的, 巨噬细胞,	未确定。包括编码免疫相关的细胞表面分子

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实例模块 I.D.	实例关键词选择	基因表达谱的评估
	树突的，炎症的，白细胞介素	的基因（CD36、CD86、LILRB）、细胞因子(IL15) 以及信号转导通路中涉及的分子（FYB、TICAM2-Toll 样受体通路）。
M 2.11	复制，抑制，RAS，自体磷酸化，致癌的	未确定。包括激酶(UHMK1、CSNK1G1、CDK6、WNK1、TAOK1、CALM2、PRKCI、ITPKB、SRPK2、STK17B、DYRK2、PIK3R1、STK4、CLK4、PKN2) 和 RAS 家族成员(G3BP、RAB14、RASA2、RAP2A、KRAS)。
M 3.1	ISRE，流感，抗病毒的，IFN-γ，IFN-α，干扰素	干扰素可诱导的：这个集合包括干扰素可诱导的基因：抗病毒的分子(OAS1/2/3/L、GBP1、G1P2、EIF2AK2/PKR、MX1、PML)，趋化因子 (CXCL10/IP-10)，信号转导分子(STAT1、STAt2、IRF7、ISGF3G)。
M 3.2	TGF-β，TNF，炎症，细胞凋亡的，脂多糖	炎症 I：包括编码涉及炎症过程的分子的基因 (如 IL8、ICAM1、C5R1、CD44、PLAUR、IL1A、CXCL16)，以及细胞凋亡的调控剂(MCL1、FOXO3A、RARA、BCL3/6/2A1、GADD45B)。
M 3.3	粒细胞，炎症，抵御，氧化，溶酶体	炎症 II：包括诱导粒细胞-巨噬细胞 CSF 或者可被粒细胞-巨噬细胞 CSF 诱导的分子(SPI1、IL18、ALOX5、ANPEP)，以及溶酶体酶 (PPT1、CTSB/S、CES1、NEU1、ASAH1、LAMP2、CAST)。
M 3.4	未提取关键词	未确定。包括蛋白质磷酸酯(PPP1R12A、PTPRC、PPP1CB、PPM1B) 和磷酸肌醇 3-激酶(PI3K) 家族成员 (PIK3CA、PIK32A、PIP5K3)。
M 3.5	未提取关键词	未确定。仅包括少量的转录物。包括血红素基因 (HBA1、HBA2、HBB)。
M 3.6	补体，宿主，氧化的，细胞骨架，T 细胞	未确定。包括 T 细胞表面标记物的大集合(CD101、CD102、CD103) 以及在血液白细胞中无所不在地表达的分子(CXRCR1：fraktalkine 受体，CD47，P-选择配体)。

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实例模块 I.D.	实例关键词选择	基因表达谱的评估
M 3.7	剪接体，甲基化，泛素，β-联蛋白	未确定。包括编码蛋白酶体亚单位的基因(PSMA2/5，PSMB5/8)；泛素蛋白连接酶 HIP2，STUB1，以及泛素连接酶复合物的组分(SUGT1)。
M 3.8	CDC，TCR，CREB，糖基化酶	未确定。包括编码几种酶的基因：氨甲基转移酶，精氨酰转移酶、天冬酰胺合成酶、甘油二酯激酶、肌醇磷酸酶、甲基转移酶、螺旋酶...
M 3.9	染色质，检查点，复制，反式激活	未确定。包括编码蛋白质激酶(PRKPIR、PRKDC、PRKCI)以及磷酸酯酶(例如 PTPLB、PPP1R8/2CB)的基因。还包括 RAS 致癌基因家族成员以及 NK 细胞受体 2B4 (CD244)。

[0071] 生物学定义

[0072] 如本文所使用的，术语“阵列”指固体的支持物或者基质，其具有连接于支持物的一种或多种肽或者核酸探针。阵列一般地具有偶联于基质表面不同已知位置的一种或多种不同的核酸或者肽探针。这些阵列，也称作“微阵列”或者“基因芯片”，其可能具有基于已知的基因组（如人类基因组）的 10,000；20000；30,000 或者 40,000 个不同的可鉴定的基因。这些阵列用于检测在样品中表达或者发现的基因的整个“转录组”或者转录池，例如，表达为 RNA、mRNA 等的核酸，其可能进行 RT 和 / 或 RT-PCR，从而产生 DNA 复制子的互补集合。可以使用机械合成方法、光诱导合成方法等加入非光刻的和 / 或光刻的方法与固相合成方法组合的方法，来制备阵列。

[0073] 描述了合成这些核酸阵列的不同技术，如在事实上任意形状的表面或者甚至多个表面上制造。阵列可以是在珠、凝胶、聚合物表面、纤维如光学纤维、玻璃或者其他合适的任意基质上的肽或核酸。阵列可以以这样的方式组装，从而可以进行诊断或者全部所包括设备的其他操作，参见例如第 6,955,788 号美国专利，其相关的部分在此结合作为参考。

[0074] 如本文所使用的，术语“疾病”指生物体具有细胞的任何异常生物状态的生理状态。疾病包括但不限于，细胞、组织、身体功能、系统或者器官的中断、停止或者失调，其可能是内在的、遗传的、感染导致的、异常细胞功能、异常细胞分裂等等所导致的。导致“疾病状态”的疾病一般地对于生物系统也即疾病的宿主是有害的。关于本发明，任何的生物状态，如感染（如病毒、细菌、真菌、寄生虫等等）、炎症、自发炎症、自身免疫、过敏反应、过敏症、癌前病变、恶性肿瘤、外科手术、移植、生理上的等等与疾病或者障碍相关的认为是疾病状态。病理状态通常与疾病状态等价。

[0075] 疾病状态也可能归类为不同级别的疾病状态。如本文所使用的，疾病或者疾病状态的级别是主观的量度，其反映疾病或者疾病状态的进展，以及在治疗前、中和后的生理反

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应。一般地，疾病或者疾病状态沿着级别或者阶段进行发展，其中疾病的影响变得越来越严重。疾病状态的级别可能由样品中细胞的生理状态所影响。

[0076] 如本文所使用的，术语“疗法”或者“治疗方案”指采取的缓和或者改变疾病状态的医学步骤，如使用药理学上的、外科的、饮食的和/或其他技术旨在减少或者消除疾病影响或症状的治疗过程。治疗方案可以包括一种或多种药物的给药或者外科手术的处方剂量。疗法在大多数情况下是有益的并降低疾病状态，但是在多种实例中疗法的效果具有不期待作用或者副作用。疗法的效果也受到宿主的生理状态的影响，所述生理状态如年龄、性别、遗传、体重、其他疾病的症状等。

[0077] 如本文所使用的，术语“药理学状态(pharmacological state)”或者“药理学状态(pharmacological status)”指这些样品，即，其将和/或已经使用一种或多种药物、外科手术等治疗，该药物、外科手术等可能影响样品中的一种或多种核酸的药理学状态，如作为药物干涉结果的新转录、稳定化和/或去稳定化的。样品的药理学状态涉及在药物治疗之前、之中和/或之后生物状态的变化，并如本文示教地可以用作诊断或者预测功能。在药物治疗或者外科手术之后的一些变化可能与疾病状态相关和/或可能与疗法的副作用无关。在药理学状态上的变化可能是下述的结果：疗法的持续时间、所处方的药物的类型和剂量、对给定治疗过程的顺从性和/或摄取的未处方的药物。

[0078] 如本文所使用的，术语“生物学状态”指为分析表达状态而分离和纯化的细胞样品的转录组(其为RNA转录物的全部集合)的状态。生物学状态通过测量细胞组分的充足性和/或活性、根据形态学表型的表征或者用于检测转录物的所述方法的组合，反映了样品中细胞的生理状态。

[0079] 如本文所使用的，术语“表达谱”指RNA的相对丰度、DNA或者蛋白质丰度或者活性水平。表达谱可以为例如转录状态或者翻译状态的量度，其通过多种方法并使用基因芯片、基因阵列、珠、多重PCR、定量PCR、连缀分析(run-on assay)、RNA印迹分析、蛋白质印迹分析、蛋白质表达、荧光活化的细胞分选(FACS)、酶联免疫吸附试验(ELISA)、化学荧光研究、酶分析、增殖研究或者任意其他方法、设备和系统的多种的任一种进行，用于确定和/或分析基因表达，上述所使用的可容易地商业获得。

[0080] 如本文所使用的，术语样品的“转录状态”包括存在于样品中的RNA种类、尤其是mRNA的性质和相对丰度。样品的整个转录状态，也即RNA的性质和丰度的组合也在此称为转录组。一般地说，测量了样品中RNA种类的整个集合的全部相对组成部分的一大部分。

[0081] 如本文所使用的，术语“模块转录载体”指反映“不同表达的基因的比例”的转录表达数据。例如，对于各个模块而言，在至少两个组之间(如健康的受试者与患者)不同地表达的转录物的比例。该载体得自两个组的样品的比较。第一分析步骤用于在各个模块中选择疾病特异性的转录集合。随后，为“表达水平”。给定疾病的组比较提供了对于各个模块不同地表达的转录物的列表。发现了不同的疾病导致不同的模块转录物的集合。随后可能以此表达水平，通过将鉴别为不同表达的疾病特异性的基因的亚集合的表达数值取平均，来计算用于单个样品的各个模块(或者多个模块)的载体。该方法使得能够产生单个样品的模块表达载体图谱，所述载体例如，本文公开的模块图谱中所描述的那些。这些载体模块图谱代表了各个模块的平均表达水平(而不是一部分不同地表达的基因)，可以获得各个样品的所述图谱。

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[0082] 使用本发明，不仅能够从模块水平上，还能在基因水平上确定并区分疾病；也即，两种疾病可能具有相同的载体（相同比例的不同地表达的转录物，相同的“极性”），然而载体的基因组成可能仍然是疾病特异性的。基因水平表达提供了明显的益处，即分析的分辨率大大提高。进一步地，本发明利用组合的转录标记物。如本文使用的，术语“组合的转录标记物”指与使用单个基因作为标记物相比，多个基因（模块的亚集合）的表达数值平均值（以及这些标记物的组成可能是疾病特异性的）。组合的转录标记物方法是唯一的，这是由于用户能够开发多变量的微阵列积分，以使用如 SLE 来评估患者的疾病严重性，或者得到本文公开的表达载体。最重要的是，已经发现了使用本发明的组合模块转录标记物，在此得到的结果在微阵列平台之间是可复制的，从而为注册审批提供了更大的可靠性。

[0083] 用于本发明的基因表达监控系统可能包括具有有限的和 / 或基础数目的基因的定制的基因阵列，所述基因是特定的和 / 或是为了一种或多种目标疾病定制的。不像一般的习惯上使用的泛基因组阵列，本发明不仅提供了这些一般的阵列在例行基因和基因组分析中的应用（不需要使用特定平台），更重要地，其提供了定制的阵列的开发，所述定制的阵列为分析提供了可选的基因集合，而不需要几千个其他不相关的基因。本发明的经优化的阵列和模块相对于现有的技术的一个明显的优点是财务成本的降低（例如每次分析的成本、材料、设备、时间、人工、培训等），更重要地，也降低了生产泛阵列的环境成本（在数据的绝大部分是不相干的情况下）。本发明的模块首次使得简单、定制的阵列的设计成为可能，该阵列使用最少数量的探针提供优化的数据，并使信噪比最大。通过减少用于分析的基因的总数，可能，例如减少制备几千个昂贵的铂掩蔽物的需要，所述铂掩蔽物用于泛基因芯片的制备中的光刻中，所述泛基因芯片产生大量不相关的数据。使用本发明，如果将本发明的有限的探针集合（或者多个集合）和用于测定和 / 或分析基因表达的下述方法或任意其他方法、设备和系统（可容易地商业获得的）一起使用，可能完全避免对微阵列的需要：例如，数码光学化学阵列、球珠阵列、珠（如 Luminex）、多重 PCR、定量 PCR、连续分析、RNA 印迹分析、甚至用于蛋白质分析如蛋白质印迹分析、2D 和 3D 凝胶蛋白质表达、MALDI、MALDI-TOF、荧光活化细胞分选 (FACS)（细胞表面或者细胞内）、酶联免疫吸附分析 (ELISA)、化学荧光研究、酶分析、增值研究。

[0084] 本发明的“分子指纹系统”可用于，在不同细胞或组织中、相同的细胞或者组织的不同的亚群体中、相同细胞或组织的不同生理状态、相同的细胞或组织的不同发育阶段，或者相同组织的不同细胞群体中，针对其他的疾病和 / 或正常的细胞对照，便于及进行表达的比较分析。在一些情况下，正常的或者野生型的表达数据可能来自在同样时间或者在同样时间附近分析的样品，或者其可能是得自或者选自现有的基因阵列表达数据库的表达数据，该数据库例如公共的数据库，如 NCBI Gene Expression Omnibus 数据库。

[0085] 如本文所使用的，术语“有差别地表达的”指在两个或者更多的样品（如疾病样品和正常样品）之间中变化的细胞成分（如核酸、蛋白质、酶的活性等等）的测量值。细胞成分可能是启动或者关闭的（存在或者不存在），与对照相比上调的或者与对照相比下调的。对于使用基因芯片或者基因阵列的应用，核酸（如 mRNA 或者其他 RNA(miRNA、siRNA、hnRNA、rRNA、tRNA 等)）的有差别的基因表达可能用于区分细胞类型或者核酸。最一般地，细胞的转录状态的测量通过定量的逆转录酶 (RT) 和 / 或定量的逆转录酶 - 聚合酶链式反应 (RT-PCR)、基因组表达分析、翻译后分析、对基因组 DNA 的修饰、易位、原位杂交等来完成

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的。

[0086] 对于一些疾病状态，可以鉴定细胞的或者形态上的差别，尤其地在疾病状态的早期水平。本发明避免了以下需要：通过考察细胞自身的基因模块或者更重要地通过考察来自免疫效应细胞的基因的细胞 RNA 表达来鉴定特异突变或者一个或多个基因，所述免疫效应细胞在其常规生理状态下作用，也即在免疫活化、免疫耐受或者甚至免疫无力过程中作用。尽管遗传突变可能导致一组基因的表达水平的显著变化，生物系统通常通过改变其他基因的表达来补偿变化。这些内在的补偿响应的结果是，许多扰动可能对于系统的可观察的表型具有很小的效果，然而对细胞组分的组成具有深远的影响。类似地，基因转录物的实际拷贝数可能不增加或者减少，然而，所述转录物的持久性或者半衰期可能受影响，这导致蛋白质产量的大幅度增长。本发明通过，在一个实施例中，观察效应细胞（如自细胞、淋巴细胞和 / 或其亚群体）而不是单个的信号和 / 或突变，消除了检测实际信号的需要。

[0087] 技术人员容易地认识到样品可能得自多种来源，包括如单个的细胞，细胞的集合，组织，细胞培养物等等。在特定的情况下，甚至可能从细胞中分离足够的 RNA，所述细胞见于例如尿液、血液、唾液、组织或者活检样品等等。在特定的情况下，可能从粘膜分泌物、粪便、眼泪、血浆、腹水、间质液、硬膜内的、脑脊液、汗或其他体液中得到足够的细胞和 / 或 RNA。核酸的来源，如来自组织或者细胞来源的，可能包括组织活检样品，一种或多种分选的细胞群，细胞培养物、细胞克隆、转化的细胞、活检样品或者单个的细胞。所述组织的来源可能包括如大脑、肝脏、心脏、肾脏、肺、脾脏、视网膜、骨骼、神经的 (neural)、淋巴节、内分泌腺、生殖器官、血液、神经 (nerve)、血管组织以及嗅上皮。

[0088] 本发明包括以下的基本组分，其可能单独使用或组合地使用，即，一个或多个数据挖掘算法；一个或多个模块水平的分析过程；对血液自细胞转录模块的表征；汇总的模块数据在人类疾病的分子诊断 / 预测的多变量分析中的应用；和 / 或模块水平的数据和结果的可视化。使用本发明，也可能开发及分析组合的转录标记物，所述标记物可能进一步地汇总于单个的多变量计分中。

[0089] 数据采集速率的爆炸刺激了用于开发微阵列数据和生物医学知识的挖掘工具和算法的发展。旨在揭开转录系统的模块组织及功能的方法包括了用于鉴定疾病的强大的分子标签的有希望的方法。实际上，该分析通过将微阵列数据概念化到超过个体基因或者系列基因的层面上，能够转变对于大规模转录研究的理解。

[0090] 本发明人认识到当前基于微阵列的研究正面临着显著的挑战，对数据的分析众所周知地“有噪音”，也即难以解释数据，并且难以在实验室及平台之间良好比较。对于微阵列数据的分析，广泛接受的方式以鉴定在研究组之间有差别地表达的基因的亚集合开始。接下来，用户随后尝试使用模式识别算法及现有的科技知识来“搞清楚”所得到的基因系列。

[0091] 本发明人开发了在分析的早期强调生物上相关基因的选择的策略，而非处理平台之间的巨大可变性。简单地说，所述方法包括了表征给定生物系统的转录组分的鉴定，，为此开发了改进的数据挖掘算法，从而从大的数据集合中分析及提取共同表达的基因的组或者转录模块。

[0092] 肺结核 (PTB) 是结核分枝杆菌 (M. tuberculosis) 引起的全球范围内发病率和死亡率主要的和增长的原因。然而，多数 M. tuberculosis 感染的个体保持为无症状的，将感染保持为潜伏性的形式，并且认为该潜伏性状态通过主动免疫响应维持。血液是免疫系统

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的管线，并因此是理想的生物材料，从血液能够建立个体的健康和免疫状态。因此，使用微阵列技术来评估血液细胞中的整个基因组的活性，我们在具有活性的肺结核和潜伏性的肺结核的患者中鉴定了不同的和相互的血液转录生物标记物标签。这些标签也不同于在对照个体中的那些。潜伏性的结核的信号，其在全血中显示出免疫细胞毒性基因表达的过表现，可能对于确定抗 M. tuberculosis 感染的保护性免疫因子有帮助，这是由于这些患者受到感染然而大多数不发展为明显的疾病。这种来自活性的和潜伏性的 TB 患者的不同的转录生物标记物标签也可用于诊断感染，并用于监测对使用抗分枝杆菌药物治疗的响应。除此之外，在活性的结核病患者中的标签能帮助确定涉及于免疫发病机理中的因子，并可能带来用于免疫治疗干预的策略。本发明涉及在先的申请，其要求了血液转录生物标记物在感染诊断中的用途的权利。然而，此在先申请未公开活性的和潜伏性的结核的生物标记物的存在，而是重点在于具有其他急性感染的儿童 (Ramilo, Blood, 2007)。

[0093] 在此对来自潜伏性的和活性的 TB 患者的血液中转录标签的鉴定可以用于测试怀疑具有结核分枝杆菌感染的患者，以及用于该疾病的健康筛查 / 早期检测。本发明还使评估对使用抗分枝杆菌药物治疗的响应成为可能。在这一背景下，在药物测试中，尤其在评估多抗药性患者的药物治疗中，试验也可能是特别有价值的。进一步地，本发明可能用于从潜伏性的结核病的免疫标签获得即时的、间隔的及长期的数据，从而更好地在接种试验中界定保护性的免疫响应。同时，在活性的结核患者中的标签能帮助确定涉及于免疫致病性的因子，并很可能带来用于免疫治疗干预的策略。

[0094] 血液代表了固有的和适应性的免疫系统的细胞的储存库和迁移区域，该细胞包括中性粒细胞、树突细胞和单核细胞或者 B 和 T 淋巴细胞，分别地在感染过程中暴露于组织中的感染性因素。因为如此，来自感染个体的全血提供了临床上相关材料的可得来源，其中使用前面所描述的用于组织内癌症 (Alizadeh AA., 2000 ; Golub, TR., 1999 ; Bittner, 2000) 和自免疫性 (Bennet, 2003 ; Baechler, EC, 2003 ; Burczynski, ME, 2005 ; Chaussabel, D., 2005 ; Cobb, JP., 2005 ; Kaizer, EC., 2007 ; Allantaz, 2005 ; Allantaz, 2007) 以及炎症 (Thach, DC., 2005) 和血液或组织中 (Bleharski, JR et al., 2003) 的感染性疾病 (Ramilo, Blood, 2007) 研究的基因表达微阵列，可以得到无偏见的分子表型。血液白细胞中基因表达的微阵列分析鉴定了诊断的和预测的基因表达标签，这对于疾病发作及对于治疗的响应的机理带来了更好的理解 (Bennet, L 2003 ; Rubins, KH., 2004 ; Baechler, EC, 2003 ; Pascual, V., 2005 ; Allantaz, F., 2007 ; Allantaz, F., 2007)。这些微阵列方法已经尝试了用于研究活性的和潜伏性的 TB，然而到现在为止只得到了少量的有差别地表达的基因 (Jacobsen, M., Kaufmann, SH., 2006 ; Mistry, R, Lukey, PT, 2007) 并且在相对少数的患者中 (Mistry, R., 2007)，其可能不足以区分其他的炎症及感染性疾病。

[0095] 为了界定 TB 的免疫标签，分析了活性的和潜伏性的 TB 患者及对照的血液；使用非常严格的临床标准选择患者。患者招募自英国伦敦，在那里活性的 TB 案例数量处于增长中，最重要的是，混淆同时感染的风险是很小的，从而得到能够区分潜伏性性的和活性 TB 的强大的标签。使用微阵列来分析整个基因组，随后的数据挖掘显示了所发现的大量基因在统计显著的水平上，在所有组的患者（包括活性的及潜伏性的患者以及健康的对照）中有差别地表达。随后，使用了基于模块数据挖掘策略的新方法，该方法为选择临床上相关的转录生物标记物提供了基础，所述生物标记物用于 SLE 及其他疾病中血液微阵列转录谱的

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分析，并改变了我们关于疾病发病的认识 (Chaussabel，2008，Immunity)。在本研究中所定义的模块图谱提供将复杂的数据组织起来并减少其维度的方式，而仍然保持了人类血液中表达的大量基因，从而可以观察特定疾病指纹 (Chaussabel，2008，Immunity)。使用此模块方法，得到了定义清晰的模块转录标签，所述转录标签在活性的和潜伏性的 TB 患者的全血中是不同的和相互的，并且还不同于健康的对照。本文描述的生物标记物改进了 PTB 的诊断，并进一步帮助定义在潜伏性的 TB 患者中抗 M. tuberculosis 的保护中重要的宿主因子，以及在活性 TB 的免疫致病中涉及的那些主因子，并从而用于减少及控制 TB 疾病。

[0096] 患者、材料及方法

[0097] 参与者募集及患者描述：参与者募集自伦敦的 St. Mary's Hospital TBClinic，Imperial College Healthcare NHS Trust，并从伦敦的 Mill Hill 的国立医学研究所 (NIMR) 的志愿者中募集健康的对照。本研究经当地位于 St MarysHospital (LREC)，London，UK 的 NHS Research Ethics Committee 核准。所有的参与者 (18 岁及以上) 给出了书面的知情同意书。在所募集的参与者确定其临时的研究分组前，满足了严格的临床标准，此后才使其分配到最终分析组中。患者及对照的群组如下：(i) 根据临床诊断并随后由结核菌培养的实验室分离 M. tuberculosis 确认活性的 PTB；(ii) 潜伏性的 TB- 由阳性的结核菌素皮肤试验确定 (TST，使用 2TU 结核菌素 (Serum Statens Institute，Copenhagen，Denmark)，如果未接种 BCG，≥ 6mm；如果接种了 BCG，≥ 15mm，同时具有使用干扰素 γ 释放分析的阳性结果 (IGRA，具体为 Quantiferon-TBGold In-tube assay，Cellestis，Australia))。该 IGRA 分析通过从全血的 IFN- γ 释放测量对抗原的反应性 (ESAT-6/CFP-10/TB 7.7- 存在于 M. tuberculosis，然而不存在于多数的环境结核杆菌中或者 M. bovis BCG 疫苗中)。

[0098] 潜伏性的 TB 患者还必须具有暴露于感染性的 TB 病例的迹象，通过亲密的居家或工作接触，或者作为病区的"新参加者"；具有偶然发现 TST 阳性而没有暴露于感染的人的迹象的患者不选入本研究。(iii) 健康的志愿者对照 (BCG 接种的和未接种的，按照 TST 分别≤ 14mm 或者≤ 5mm；以及按照 IGRA 阴性的)。怀孕、已知免疫抑制、进行免疫抑制疗法或者有糖尿病或者自身免疫疾病的患者也无选入资格并从开始的研究中排除。HIV 阳性的个体 (伦敦的 TB 患者仅有 1％具有此前未诊断的 HIV) 从研究中排除。在给予任何抗分枝杆菌药物之前，并随后在设定的时间间隔为了本研究收集来自活性的及潜伏性的 PTB 患者的血液，作为随后的研究的纵向部分。

[0099] 对每位参与者，收集预期的详细的临床信息，并将其输入到本发明人开发的可经网络访问的数据库中。使用该记录的临床数据，以及前文所述的基于免疫的分析，将 58 名参与者中的 15 人从本研究中排除，这是由于其不满足本研究的标准准则。这产生了下述群体：6 名未接种 BCG 的健康志愿者；6 名接种 BCG 的健康志愿者，17 名潜伏性的 TB 患者及 14 名活性的 PTB 患者，将所有的这些样品用于 RNA 分离。在处理进行之后，来自活性 TB 患者的一个样品未产生足够的除去球蛋白的 RNA，故将其从最终的分析中排除。

[0100] 用于微阵列的 RNA 采样，提取，处理：将来自上述患者群体的全血采集到 Tempus 试管 (Applied Biosystems，Foster City，CA，USA) 中，并于 RNA 提取之前储存于 -20℃到 -80℃下。使用 PerfectPure RNA 血液试剂盒 (5PRIME Inc，Gaithersburg，MD，USA) 提取总 RNA。将样品用 100％冷乙醇匀浆，涡旋震荡，随后在 4000g 下在 0℃下离心 60 分钟，去除上清。将 300μl 裂解溶液加入沉淀中并进行涡旋震荡。随后根据生产商的说明进行

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RNA 结合，Dnase 处理，洗涤及 RNA 洗脱步骤。经分离的总 RNA 使用 GLOBINclearTM96- 孔格式化试剂盒 (Ambion，Austin，TX，USA) 根据生产商的说明除去球蛋白。总的及除去球蛋白的 RNA 完整性使用 Agilent 2100 Bioanalyzer(Agilent，Palo Alto，CA) 进行评估。在处理进行后，来自活性的 TB 患者的一个样品未得到足量的除去球蛋白的 RNA，并因此从最终的分析中排除。随后从所述除去球蛋白的 RNA 使用 Illumina CustomPrep RNA 扩增试剂盒 (Ambion，Austin，TX，USA) 制备生物素化的扩增的 RNA 靶 (cRNA)。按照生产商的规程，将标记的 cDNA 在 Sentrix Human-6 V2 BeadChip 阵列 (> 48，000 个探针，Illumina Inc，San Diego，CA，USA) 上杂交过夜，洗涤，封闭，染色并在 Illumina BeadStation 500 上扫描。使用 Illumina's BeadStudio 的第 2 版软件来从扫描中产生信号强度数值，扣除背景，并将各个微阵列与所有样品 (每个芯片的校正) 的中间平均强度进行比较。该经校正的数据用于所有随后的数据分析。

[0101] 微阵列数据分析：使用基因表达分析软件程序，Genespring 7.1.3 版 (Agilent) 来进行样品的统计分析及分级聚类。按照在结果和图示部分所述地，将有差别地表达的基因选出并聚类。

[0102] 结果和讨论

[0103] 血液标签将活性的和潜伏性的 TB 患者彼此分开，并从健康的对照个体区分开：为了确定来自活性的和潜伏性的 TB 患者的血液样品是否携带使活性的和潜伏性的 TB 与健康对照相比能够区分的基因表达标签，进行了分步的分析。在滤除了偏离中位数小于 2 倍 (即，具有平坦的图谱) 的未检测的转录物及基因之后，通过得自全血 RNA 样品 (来自活性的及潜伏性的 TB 及健康的对照) 的表达谱的皮尔逊相关系数对 6269 个基因进行无人监管的聚类分析 (图 1)。所述无人监管的分析确定了不同的标签，该标签发现对应于不同的临床表型：在具有活性肺部 TB 的患者 (活性的 PTB) 中；以及在具有潜伏性的结核病的个体 (潜伏性的 TB) 中。样品的分组不是完美的 (13 名具有活性的患者的 10 名，以及 17 名具有潜伏性 TB 的患者的 11 名)。然而，在来自患者的训练集合的该组中的大部分活性的 PTB 及潜伏性的 TB 患者显示出具有清楚及不同的转录标签。重要的是，这些标签显示出表现在本研究所选择的人量种族中，包括白人，非洲黑人，亚洲印第安人，亚洲孟加拉人，亚洲其他人种，爱尔兰白人，混血白人，加勒比海黑人 (这些数据的细节未显示。)

[0104] 将 6269 个基因的列表进一步使用非参数的统计组比较 (Kruskal-Wallis 试验) 进行分析，从而鉴定在组之间有差别地表达的基因。使用适度严格的多点比较校正法来控制 I 型误差 (Benjamini-Hochberg 校正)，在活性的 TB 和潜伏性的 TB 以及健康的对照之间有 1473 个基因有差别地表达 / 表现 (P < 0.01)(图 2；以及随同此文件递交的长表格中列举的 1473 个基因)。这些聚类的基因随后与文献中的相关发现进行联系。对于本体论术语“免疫响应”，这些基因的过滤产生了一列 158 个这类基因 (图 3A-D；表 2)。158 个基因的表达 / 表现谱 (图 3A-3D) 使活性的 TB 患者从潜伏性的 TB 患者及从健康的对照个体能区分出来。

[0105] 表 2. 标注了基因本体论术语的生物过程的 158 个基因的列表：免疫响应，及发现在活性的 TB 及其他的临床组之间有差别地表达的 (p < 0.01)

[0106]

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基因符号	描述
LILRB3	白细胞免疫球蛋白-样受体，亚家族 B (具有 TM 以及 ITIM 功能域)，成员 3
PGLYRP1	肽聚糖识别蛋白 1
FAS	Fas (TNF 受体超家族，成员 6)
IFITM3	干扰素诱导的跨膜蛋白 3 (1-8U)
FCGR2A	IgG 的 Fc 片段，低亲和性 IIa，受体 (CD32)
FCGR2A	IgG 的 Fc 片段，低亲和性 IIa，受体 (CD32)
ST6GAL1	ST6 β-半乳糖酰胺 α-2,6-唾液酸转移酶 1
ETS1	v-ets 骨髓成红血细胞增多症病毒 E26 致癌基因类似物 1 (鸟类的)
CYBB	细胞色素 b-245，β 多肽 (慢性肉芽肿病)
IFNAR1	干扰素 （α，β 和 ω) 受体 1
LY96	淋巴细胞抗原 96
TRIM22	三结构域蛋白家族 22
GBP2	鸟苷酸结合蛋白 2,干扰素可诱导的
DDX58	DEAD (Asp-Glu-Ala-Asp) 盒多肽 58
LAX1	淋巴细胞跨膜接头 1
IFI16	干扰素，γ 可诱导蛋白 16
LCK	淋巴细胞特异的蛋白质酪氨酸激酶
IL32	白细胞介素 32
CXCL16	趋化因子 (C-X-C 基序) 配体 16
CD40LG	CD40 配体(TNF 超家族，成员 5，超-IgM 综合征)
TNFSF13B	肿瘤坏死因子 (配体) 超家族，成员 13b
IRF2	干扰素调节因子 2
C5	补体成分 5

[0107]

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基因符号	描述
CD46	CD46 分子，补体调节蛋白
TNFAIP6	肿瘤坏死因子，α 诱导蛋白 6
DPP4	二肽基肽酶 4 (CD26，腺苷脱氨基酶复合蛋白 2)
EBI2	埃-巴二氏病毒诱导的基因 2 (淋巴细胞特异性的 G 蛋白偶联受体)
NFX1	核转录因子，X-盒结合 1
MICB	MHC I 型多肽相关序列 B
GBP3	鸟苷酸结合蛋白 3
SLAMF7	SLAM 家族成员 7
CARD12	NLR 家族，含有 CARD 功能域 4
GBP6	鸟苷酸结合蛋白家族，成员 6
IFIT3	具有三十四肽重复 3 的干扰素-诱导的蛋白
TAP2	转运子 2，ATP-结合盒，亚家族 B (MDR/TAP)
HLA-DPB1	主要组织相容性复合体，II 类，DP β 1
CD3G	CD3g 分子，γ (CD3-TCR 复合物)
PRKCQ	蛋白激酶 C，θ
IL7R	白细胞介素 7 受体
SLAMF1	信号转导淋巴细胞活化分子家族成员 1
CD274	CD274 分子
GBP1	鸟苷酸结合蛋白 1，干扰素-可诱导的，67kDa
IFITM2	干扰素诱导的跨膜蛋白 2 (1-8D)
ITK	IL2-可诱导的 T-细胞激酶
APOL2	载脂蛋白 L，2
PSME1	蛋白酶体 (前体，巨蛋白因子) 活化剂亚单位 1 (PA28 α)
LAT2	T 细胞家族活化的连接蛋白，成员 2
IL18RAP	白细胞介素 18 受体附属蛋白
OSM	制瘤蛋白 M
CD6	CD6 分子
WWP1	含有 WW 功能域的 E3 泛素蛋白连接酶 1
CD3E	CD3e 分子，epsilon (CD3-TCR 复合物)

[0108]

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基因符号	描述
VIPR1	血管活性肠肽受体 1
TNFSF10	肿瘤坏死因子 (配体) 超家族, 成员 10
PRKRA	蛋白质激酶, 干扰素可诱导的双链 RNA 依赖的激活剂
TNFRSF1A	肿瘤坏死因子受体超家族, 成员 1A
BCL6	B-细胞 CLL/淋巴瘤 6 (锌指蛋白 51)
IL8	白细胞介素 8
OAS3	2'-5'-寡腺苷酸合成酶 3, 100kDa
IFIH1	由螺旋酶 C 功能域 1 诱导的干扰素
SIGIRR	单一免疫球蛋白和 toll-白细胞介素 1 受体 (TIR)功能域
SIGIRR	单一免疫球蛋白和 toll-白细胞介素 1 受体 (TIR)功能域
SIT1	信号转导域调控跨膜接头 1
ITGAM	整合素, α M (补体成分 3 受体 3 亚单位)
C1QB	补体成分 1, q 亚成分, B 链
IL27RA	白细胞介素 27 受体, α
ALOX5AP	花生四烯酸酯 5-脂肪氧合酶-活化蛋白
SERPING1	丝氨酸蛋白酶抑制蛋白肽酶抑制剂, 分化枝 G (C1 抑制剂), 成员 1, (血管性水肿, 遗传的)
IL1RN	白细胞介素 1 受体拮抗物
IL1RN	白细胞介素 1 受体拮抗物
CLEC4D	C-型凝集素功能域家族 4, 成员 D
ICOS	可诱导的 T 细胞共刺激分子
OAS1	2',5'-寡腺苷酸合成酶 1, 40/46kDa
ZAP70	zeta-链 (TCR) 关联的蛋白质激酶 70kDa
IL1B	白细胞介素 1, β
C4BPA	补体成分 4 结合蛋白 α
TNFSF13	肿瘤坏死因子 (配体) 超家族, 成员 13
IFI30	干扰素, γ -可诱导蛋白 30
HPSE	肝素酶

[0109]

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基因符号	描述
CD59	CD59 分子，补体调节蛋白
CTLA4	细胞毒素的 T-淋巴细胞关联蛋白 4
BCL2	B-细胞 CLL/淋巴瘤 2
TNFRSF7	CD27 分子
FPR1	甲酸基肽受体 1
IL2RA	白细胞介素 2 受体，α
GATA3	GATA 结合蛋白 3
S100A9	S100 钙结合蛋白 A9
TLR8	toll-样受体 8
NCF1	嗜中性粒细胞胞液因子 1，(慢性肉芽肿病，常染色体 1)
BCL6	B-细胞 LL/淋巴瘤 6 (锌指蛋白 51)
BST1	骨髓基质细胞抗原 1
G1P2	ISG15 泛素样修饰剂
C1QA	补体成分 1，q 亚成分，A 链
TCF7	转录因子 7 (T 细胞特异的，HMG-盒)
IFITM1	干扰素诱导的跨膜蛋白 1 (9-27)
TAPBPL	TAP 结合蛋白样
AIM2	在黑色素瘤 2 中缺失
CCR7	趋化因子 (C-C 基序) 受体 7
LTBR	淋巴毒素 β 受体 (TNFR 超家族，成员 3)
FYB	FYN 结合蛋白(FYB-120/130)
NFIL3	核因子，白细胞介素 3 调控的
LAT	用于 T 细胞活化的连接物
CBLB	Cas-Br-M (鼠) 同向性逆转录病毒转化序列 b
CD74	CD74 分子，主要组织相容性复合体，II 型不变的链
TAP2	转运子 2，ATP-结合盒，亚家族 B (MDR/TAP)
FLJ14466	跨膜蛋白 142A
PSMB9	蛋白酶体 (前体，巨蛋白因子) 亚单位，β型，9 (大的多功能肽酶 2)
PSMB8	蛋白酶体 (前体，巨蛋白因子) 亚单位，β型，8 (大的多功能肽酶

[0110]

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基因符号	描述
	7)
FAIM3	Fas 细胞凋亡抑制分子 3
LTA4H	白细胞三烯 A4 水解酶
IRF1	干扰素调控因子 1
OAS2	2'-5'-寡腺苷酸合成酶 2, 69/71kDa
RELB	v-rel 网织内皮组织增殖性病毒致癌基因类似物 B, B 细胞 3(鸟类的)中的 κ 轻多肽基因增强子的核因子
TRA@	T 细胞受体 α 基因座
LTB4R	白细胞三烯 B4 受体
PIK3R1	磷酸肌醇-3-激酶, 调节亚单位 1 (p85 α)
OASL	2'-5'-寡腺苷酸合成酶-样
OASL	2'-5'-寡聚腺苷酸合成酶-样
PSME2	蛋白酶体 (前体, 巨蛋白因子) 活化剂亚单位 2 (PA28 β)
CLEC6A	C-型凝集素功能域家族 6, 成员 A
NBN	断蛋白
FCGR1A	IgG 的 Fc 片段, 高亲和性的 Ia, 受体 (CD64)
SH2D1A	SH2 功能域蛋白 1A, 邓肯氏病 (淋巴细胞增生综合征)
IL15	白细胞介素 15
LY9	淋巴细胞抗原 9
LILRB1	白细胞免疫球蛋白-样受体, 亚家族 B (具有 TM 和 ITIM 功能域), 成员 1
APOL3	载脂蛋白 L, 3
PSMB8	蛋白酶体 (前体, 巨蛋白因子) 亚单位, β 型, 8 (大的多功能的肽酶 7)
CCR6	趋化因子 (C-C 基序) 受体 6
PDCD1LG2	程序性细胞死亡 1 配体 2
CD96	CD96 分子
EPHX2	环氧化氢水解酶 2, 细胞质的
BST2	骨髓基质细胞抗原 2
RIPK2	受体相互作用丝氨酸-苏氨酸激酶 2
SCAP1	src 激酶关联的磷蛋白质 1

[0111]

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基因符号	描述
GBP5	鸟苷酸结合蛋白 5
TRAT1	T 细胞受体相关的跨膜接头 1
ALOX5	花生四烯酸酯 5-脂氧合酶
LY9	淋巴细胞抗原 9
TAP1	转运子 1, ATP-结合盒,亚家族 B (MDR/TAP)
RHOH	ras 类似物基因家族, 成员 H
IFI35	干扰素诱导的蛋白 35
CD28	CD28 分子
FYB	FYN 结合蛋白(FYB-120/130)
IFIT2	具有三十四肽重复 2 的干扰素诱导蛋白
TLR7	toll-样受体 7
CD2	CD2 分子
FCER1G	IgE 的 Fc 片段, 高亲和性的 I, 受体用于; γ 多肽
SMAD3	SMAD 家族成员 3
FCER1A	IgE 的 Fc 片段, 高亲和性的 I, 受体用于; α 多肽
SERPINA1	丝氨酸蛋白酶抑制蛋白肽酶抑制剂, 分化枝 A (α-1 抗蛋白酶, 抗胰岛素), 成员 1
SERPINA1	丝氨酸蛋白酶抑制蛋白肽酶抑制剂, 分化枝 A (α-1 抗蛋白酶, 抗胰岛素), 成员 1
SECTM1	分泌的及跨膜 1
NMI	N-myc (和 STAT) interactor
TLR5	toll-样受体 5
IFIT3	具有三十四肽重复 3 的干扰素诱导蛋白
IFIT3	具有三十四肽重复 3 的干扰素诱导蛋白
CD5	CD5 分子

[0112] 在活性的 TB 中过表达 / 表现的基因：感兴趣的是大量 IFN- 相关的 / 可诱导的基因被表达：例如干扰素 (IFN)- 可诱导的基因，如 SOCS1、STAT1、PML(TRIM19)、TRIM22、多种鸟苷酸结合蛋白以及多种其他的表 2 中所标出的 IFN- 可诱导的基因，然而有趣的是，这些在潜伏性的 TB 患者中是不明显的，尽管这些患者在全血中的 IFN- γ 转录物的表现 / 表达实际上比活性的 TB 患者更高。为了关注这一点，特定家族的基因（部分基因已知由 IFN 上调，而其他的则不）进行进一步的研究，包括 TRIM 家族。

[0113] TRIMS 的亚集合在活性的 TB 中过表达 / 表现：蛋白质的三结构域蛋白 (TRIM) 家族特征在于保守的结构 (Reymond, A., EMBO J., 2001) 并已经显示出具有多种功能，包括 E3 泛素连接酶活性，诱导细胞增殖、分化和凋亡，免疫细胞信号转导 (Meroni, G., Bioessays,

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2005)。它们的参与牵涉到蛋白质－蛋白质相互作用，自身免疫以及发育 (Meroni, G., Bioessays, 2005)。进一步地，许多 TRIM 蛋白据发现具有抗病毒活性，并可能地牵涉到先天免疫 (Nisole, F, 2005, Nat. Rev. Microbiol. ;Gack, MU., 2007, Nature)。有趣的是，30 种 TRIM 转录物（一些重叠的探针）显示在活性的 TB 中表达，一些也在潜伏性的 TB 及健康的对照血液中表达（图 4 ;表 3)。这些 TRIM 中的大多数此前显示在人类白噬细胞和小鼠白噬细胞及树突细胞中表达 (Rajsbaum, 2008, EJI ;Martinez, FO., J. Imm., 2006)，并由 IFN 所调控，然而显示出在 DC 或者在 T 细胞中必要地表达的 TRIM(Rajsbaum, 2008, EJI) 未被检测到，或者未发现相对于健康的对照血液，在活性的或者潜伏性的 TB 中有差别地表达。有趣的是，发现了 TRIM 5、6、19(PML)、21、22、25、68 过表现 / 表达；尽管其他的低表现 / 表达：TRIM 28、32、51、52、68。感兴趣的是一组 TRIM 在活性的 TB 中高度表达，然而在潜伏性的 TB 及健康的对照中低到无法检测，并且它们中的四个 (TRIM 5、6、21、22) 已经显示集中在人类染色体 11 上，并据报道具有抗病毒的活性 (Song, B., 2005, J. Virol ;Li, X, Virology, 2007)。然而发现了相对于潜伏性的 TB 及健康的对照，一组 TRIM 在活性的 TB 患者的血液中低表达，其包括 TRIM 28、32、51、52、68 以及根据报道这些或者不在得自人类血液的白噬细胞中表达 (TRIM 51)，或者仅仅在未分化的单核细胞 (TRIM-28、52) 或者未活化的白噬细胞或者交替活化的白噬细胞 (TRIM-32) 中表达，或者在来自人类血液的经分化的活化白噬细胞中仅仅上调到低的水平 (TRIM-68) (Martinez, FO., J. Imm., 2006)。

[0114] 表 3. 在活性的肺结核、潜伏性的结核和健康的对照中有差别地表达的 TRIM 基因

[0115]

普通名称	基因符号	描述
RNF94; STAF50; GPSTAF50	TRIM22	三结构域蛋白 22
RNF91; SPRING; KIAA0282	TRIM9	三结构域蛋白 9
MYL; RNF71; PP8675; TRIM19	PML	早幼粒细胞性白血病
RNF89	TRIM6	三结构域蛋白 6
TRIM51;	TRIM51	含有 SPRY 功能域的 5

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普通名称	基因符号	描述
MGC10977		
RNF9; HERF1; RFB30; MGC141979	TRIM10	三结构域蛋白 10
PML	PML	早幼粒细胞性白血病; 同义词: MYL, RNF71, PP8675, TRIM19; 同工型 7 由转录物变体 7 编码; 早幼粒细胞性白血病, 诱导子; 三结构域蛋白 TRIM19; 早幼粒细胞性白血病蛋白质;智人早幼粒细胞性白血病 (PML), 转录物变体 7, mRNA.
RNF88; TRIM5α	TRIM5	三结构域蛋白 5
RNF88; TRIM5α	TRIM5	三结构域蛋白 5
BIA2; DKFZp434C091	TRIM58	三结构域蛋白 58
Trif; HSD34; RNF36	TRIM69	三结构域蛋白 69
RNF88; TRIM5α	TRIM5	三结构域蛋白 5
SSA; RO52; SSA1; RNF81	TRIM21	三结构域蛋白 21
KIAA0129	TRIM14	三结构域蛋白 14
RNF9; HERF1; RFB30; MGC141979	TRIM10	三结构域蛋白 10
EFP; Z147; RNF147; ZNF147	TRIM25	三结构域蛋白 25
HLS5; MAIR; KIAA1098; MGC17233	TRIM35	三结构域蛋白 35
RNF86; KIAA0517	TRIM2	三结构域蛋白 2
RNF9; HERF1; RFB30; MGC141979	TRIM10	三结构域蛋白 10

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普通名称	基因符号	描述
GNIP; RNF90	TRIM7	三结构域蛋白 7
KIAA0129	TRIM14	三结构域蛋白 14
TRIM50B; MGC45477	TRIM50B	三结构域蛋白 73
4732463G12Rik	TRIM65	三结构域蛋白 65
MRF1; TSBF1; RNF104; TRIM57; MGC26631; MGC129860; MGC129861	TRIM59	三结构域蛋白 59
FMF; MEF; TRIM20; MGC126560; MGC126586	MEFV	地中海热
TRIM52		三结构域蛋白 52
CAR; LEU5; RFP2; DLEU5; RNF77	RFP2	三结构域蛋白 13
KAP1; TF1B; RNF96; TIF1B; FLJ29029	TRIM28	三结构域蛋白 28
SS-56; RNF137; FLJ10369; MGC126176	TRIM68	三结构域蛋白 68
HT2A; BBS11; TATIP; LGMD2H	TRIM32	三结构域蛋白 32

[0118] 在活性的 TB 患者中特定的免疫调节配体的选择性过表达 / 表现：对不同的转录谱的分析显示来自基因 CD274 (PDL1) 和 PCDLG2 (PDL2, CD273) 的转录物仅仅在活性的 TB 患者中表达（图 5A 及 B）。这些分子已经此前显示出牵涉到对急性和慢性的病毒感染的免疫响应的调控中 (A Sharpe, Ann. Rev. Imm.)。在 T 细胞和 APC 相互作用中，这些分子作为对于分子 PD1 的抑制性共刺激受体起作用，并且该通路的阻碍显示出恢复了在 HIV，乙肝和丙肝感染中抗原特异的 T 细胞的增殖和效应功能。

[0119] 在活性的 TB 中低表达 / 表现的基因：引人注目的是，已知在 T 细胞中表达的多种

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基因（一些也在 NK 及 B 细胞上表达）发现在活性的 TB 患者的血液中极度下调 / 低表现（图 3D），（然而不在潜伏性的 TB 或者健康的对照中，其包括 CD3，CTLA-4，CD28，ZAP-70（T，NK 和 B 细胞），IL-7R，CD2（也在 B 细胞上），SLAM（也在 NK 细胞上），CCR7，GATA-3（也在 NK 细胞中）。这可能意味着在 T，NK 和 B 细胞中，在活性的 PTB 期间基因表达下调，或者细胞已经由于 M. tuberculosis 的感染在别处（如肺）重新募集。现在对来自不同患者组的血液采用流式细胞分析进行研究，同时也通过对来自不同患者组的 T 细胞的经纯化的群落的转录分析。

[0120] 相对于健康的对照，在潜伏性的和活性的患者中对转录谱的更加严格的统计分析。此前通过鉴定在各组之间有差别地表达的基因，利用非参数的 Kruskal-Wallis 试验进一步进行了统计组比较，然而现在为了控制 I 型误差，使用最严格的多重比较校正（Bonferroni 校正）。在这种增加的严格性之下，有 46 个基因（$P < 0.1$）和 18 个基因（$P < 0.05$）被鉴定为在各组之间有差别地表达（图 6 和 7；表 4 和 5）。在所述 46 个基因中，在活性的 TB 患者的血液中大量的 IFN 可诱导的基因如 STAT-1、GBP 和 IRF-1 仍然观察到是过表达 / 表现的，并且在潜伏性的患者或健康对照中为下调或不改变。在活性的 TB 患者的血液中，也发现大量的这些基因为过表达 / 表现的，甚至在严格性最高的分析中仍然提取出基因（Bonferroni 校正，< 0.05）。在活性的 TB 中观察到 46 个基因的组中，仅有 3 个转录物下调 / 低表现，其包括 IL-7R（在 T 细胞中表达），趋化因子受体 CXCR3（在更高的统计严格性下丢失）和 α 1- 血影蛋白。对 CXCR3 的低表达 / 表现感兴趣，这是由于已表明该趋化因子受体在 Th1 细胞中高度地表达，所述 Th1 细胞在抗分枝杆菌感染的保护中是需要的，这可能反映它们抑制或者迁移出血液到达受感染的组织。表 5 包括 18 个基因，其中在活性的 PTB 中 IL7R 和 SPTAN1 低表现 / 表达，其他所有的过表现 / 表达并且对于活性的疾病是诊断性的。

[0121] 表 4 在活性的 • TB 和其他的临床组之间有差别地表达的基因。

[0122]

基因符号	描述
FAM84B	具有序列相似性 84 的家族，成员 B
CXCR3	趋化因子 (C-X-C 基序) 受体 3
ETV7	Ets 变体基因 7 (TEL2 致癌基因)
DUSP3	双特异性磷酸酶 3 (痘苗病毒磷酸酶 VH1-相关的)
WARS	色氨酰基-tRNA 合成酶

[0123]

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基因符号	描述
CNIH4	cornichon 类似物 4 (果蝇)
STAT1	转录 1 的信号转导器及活化剂, 91kDa
IRF1	干扰素调节因子 1
LILRB1	白细胞免疫球蛋白-样受体, 亚家族 B (具有 TM 和 ITIM 功能域), 成员 1
SIPA1L1	信号诱导的增殖相关的 1 样 1
GSDMDC1	含有 gasdermin 功能域的 1
DYNLT1	动力蛋白, 轻链, Tctex-类型 1
DKFZp761E198	DKFZp761E198 蛋白
LOC400759	
GBP1	鸟苷酸结合蛋白 1, 干扰素可诱导的, 67kDa
GBP5	鸟苷酸结合蛋白 5
FLJ11259	损伤调控的自噬调节蛋白
LYPLA1	溶血磷脂酶 I
RHBDF2	菱形体 5 类似物 2 (果蝇)
PLEK	普列克底物蛋白
ANKRD22	锚蛋白重复功能域 22
CASP1	胱天蛋白酶 1, 细胞凋亡相关的半胱氨酸肽酶 (白细胞介素 1, β, 转化酶)
FLJ39370	染色体 4 开放阅读框 32
FBXO6	F-盒蛋白 6
GCH1	GTP 环水解酶 1 (多巴-响应的肌力失常)
GBP4	鸟苷酸结合蛋白 4
IFI30	干扰素, γ-可诱导的蛋白 30
VAMP5	囊泡关联的膜蛋白 5 (肌短蛋白)
GBP2	鸟苷酸结合蛋白 2, 干扰素-可诱导的
STX11	突触融合蛋白 11
SPTAN1	血影蛋白, α, 非红细胞的 1 (α-胞衬蛋白)
POLB	聚合酶 (DNA 定向的), β
IL7R	白细胞介素 7 受体
APOL6	载脂蛋白 L, 6
ATG3	ATG3 自噬相关的 3 类似物 (酿酒酵母)

[0124]

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基因符号	描述
SQRDL	硫醌还原酶样 (酵母)
PSME2	蛋白酶体 (前体, 巨蛋白因子) 活化剂亚单位 2 (PA28 β)
FLJ10379	S1 RNA 结合功能域 1
WDFY1	包括 WD 重复和 FYVE 功能域的 1
TAP2	转运子 2, ATP-结合盒,亚家族 B (MDR/TAP)
NPC2	尼曼-皮克病, 类型 C2
ATF3	活动的转录因子 3
VAMP3	囊泡关联的膜蛋白 3 (小细胞小泡蛋白)
PSMB8	蛋白酶体 (前体, 巨蛋白因子) 亚单位, β 型, 8 (大的多功能的肽酶 7)
JAK2	Janus 激酶 2 (蛋白质酪氨酸激酶)

[0125] 表 5. 在活性的 TB 和其他的临床组之间显著不同地表达的 18 个基因。

[0126]

基因符号	描述
VAMP5	囊泡关联的膜蛋白 5 (肌短蛋白)
GBP2	鸟苷酸结合蛋白 2, 干扰素-可诱导的
STX11	突触融合蛋白 11
SPTAN1	血影蛋白, α, 非红细胞的 1 (α-胞衬蛋白)
POLB	聚合酶 (DNA 指向的), β
IL7R	白细胞介素 7 受体
APOL6	载脂蛋白 L, 6
ATG3	ATG3 自噬相关的 3 类似物 (酿酒酵母)
SQRDL	硫醌还原酶样 (酵母)
PSME2	蛋白酶体 (前体, 巨蛋白因子) 活化剂亚单位 2 (PA28 β)
FLJ10379	S1 RNA 结合功能域 1
WDFY1	含有 WD 重复和 FYVE 功能域的 1
TAP2	转运子 2, ATP-结合盒,亚家族 B (MDR/TAP)
NPC2	尼曼-皮克病, 类型 C2
ATF3	活化转录因子 3
VAMP3	囊泡关联的膜蛋白 3 (小细胞小泡蛋白)
PSMB8	蛋白酶体 (前体, 巨蛋白因子) 亚单位, β 型, 8 (大的多功能的肽酶 7)
JAK2	Janus 激酶 2 (蛋白质酪氨酸激酶)

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[0127] 在具有活性的和潜伏性的 TB 的患者和健康的对照之间改进的识别：前述的方法尽管能够从潜伏性的 TB 和健康的对照中识别活性的 TB，但是较不能在全部三个临床组之间识别。为了选择识别基因，使用了随后的方法。首先，在来自健康的个体的血液中表达的基因与潜伏性的 TB 患者进行比较，其在 $p < 0.005$ 下使用 Wilcoxon-Mann-Whitney 试验，得到了 89 个有识别力的基因。随后在 $p < 0.5$ 下再次使用 Wilcoxon-Mann-Whitney 试验以及最严格的 Bonferroni 相关系数将来自健康的个体的血液中表达的基因与活性的 TB 患者进行比较，得到了一组 30 个有识别力的基因。该列表合并得到总计 119 个的识别基因（表 6）。随后使用通过皮尔逊相关系数进行的无人监督的聚类分析，将该列基因用于审查全部临床组的数据集。该分析产生三个不同的临床组聚类（图 8A 到 8F）：一个聚类由 13 名活性的 TB 患者中的 11 名组成（图 8，聚类 C）；第二个聚类由 17 名潜伏性的 TB 患者中的 16 名和 1 名活性的 TB 患者组成（图 8，聚类 B）；第三个聚类含包括于本研究中的全部 12 名健康对照，外加 1 名活性的 TB 以及 1 名潜伏性的 TB 落单者（图 8，聚类 A）。对于图 8A 到 8F 中的各个，患者 / 临床组的聚落水平地表示，基因的聚落竖直地表示。全部 119 个基因的表达 / 表现谱的形式（图 8A）现在使得所有三个临床组能从彼此识别出来：也即，使得活性 TB，潜伏性的 TB 和健康的个体彼此识别，各个临床组显示这些 119 个基因或者其亚组的独特的表达 / 表现谱。技术人员应该认识到可以将 1、2、3、4、5、6、7、8、10、12、15、20、25、30、35 或者更多的基因置于代表基因聚类的数据集中，所述基因可以单独地或者与其他的聚类共同在临床组 A（健康的），B（潜伏性的），C（活性的）的聚落之间比较，各个临床组可以显示得自这些 119 个基因的独特的表达 / 表现谱。

[0128] 具体地，图 8B 证明了基因 ST3GAL6、PAD14、TNFRSF12A、VAMP3、BR13、RGS19、PILRA、NCF1、LOC652616、PLAUR(CD87)、SIGLEC5、B3GALT7、IBRDC3(NKLAM)、ALOX5AP(FLAP)、MMP9、ANPEP(APN)、NALP12、CSF2RA、IL6R(CD126)、RASGRP4、TNFSF14(CD258)、NCF4、HK2、ARID3A、PGLYRP1(PGRP) 在潜伏性的 TB 患者的血液中低表达 / 低表现，而在健康的个体或者活性的 TB 患者的血液中不如此。

[0129] 表现于图 8C 中的基因 ABCG1、SREBF1、RBP7(CRBP4)、C22orf5、FAM101B、S100P、LOC649377、UBTD1、PSTPIP-1、RENBP、PGM2、SCLF2、FAM7A1、HOM-TES-103、NDUFAF1、CES1、CYP27A1、FLJ33641、GPR177、MID1IP1(MIG-12)、PSD4、SF3A1、NOV(CCN3)、SGK(SGK1)、CDK5R1、LOC642035 显示出在健康对照个体的血液中过表达 / 过表现，而在潜伏性的 TB 患者的血液中低表达 / 低表现，并在很大程度上在活性的 TB 患者的血液中低表达 / 低表现。

[0130] 在图 8D 中，ARSG、LOC284757、MDM4、CRNKL1、IL8、LOC389541、CD300LB、NIN、PHKG2、HIP1 的基因谱在健康个体的血液中显示为过表达 / 过表现，然而在潜伏性的及活性的 TB 患者的血液中低表达 / 低表现。相反地，在图 8D 中的基因 PSMB8(LMP7)、APOL6、GBP2、GBP5、GBP4、ATF3、GCH1、VAMP5、WARS、LIMK1、NPC2、IL-15、LMTK2、STX11(FHL4) 在活性 TB 的血液中显示为过表达 / 过表现，然而在潜伏性的 TB 患者以及健康的对照个体中低表达 / 低表现。

[0131] 在图 8E 中，FLJ11259(DRAM)、JAK2、GSDMDC1(DF5L)(FKSG10)、SIPA1L1、[2680400](KIAA1632)、ACTA2(ACTSA)、KCNMB1(SLO-B) 的基因谱全部在来自活性的 TB 患者的血液中过表达 / 过表现，然而在来自潜伏性的 TB 患者和健康的对照个体的血液中未表现或者甚至低表达 / 低表现。与此相反地，基因 SPTAN1、KIAA0179(Nnp1)(RRP1)、FAM84B(NSE2)、

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SELM、IL27RA、MRPS34、[6940246](IL23A)、PRKCA(PKCA)、CCDC41、CD52(CDW52)、[3890241](ZNF404)、MCCC1(MCCA/B)、SOX8、SYNJ2、FLJ21127、FHIT 在活性的 TB 患者中的血液中低表达/低表现，然而在潜伏性的 TB 患者或者健康的对照个体中不如此，在此它们过表达/过表现。

[0132] 列于图 8D 和 8E 中的多个（在按照前述的本方法选择的 119 个基因内）在活性的 TB 患者的血液中发现过表达/过表现的这些基因对于在活性的、潜伏性的 TB 患者以及健康的对照中，通过前文描述的使用转录谱的更强严格性分析的可选方法所鉴定的那些而言是常见的（在图 8D 和图 8E 中表示为下划线的基因包括于图 7、表 5 的基因列表中，18 个基因，$p < 0.05$；图 8D 及 8E 中表示为斜体的基因包括于图 6，表 4 的基因列表中，46 个基因，$P < 0.1$）。

[0133] 在图 8F 中显示 CD52(CDW52)、[3890241](ZNF404)、MCCC1(MCCA/B)、SOX8、SYNJ2、FLJ21127、FHIT 的基因谱在活性的 TB 患者的血液中低表达/低表现，然而在潜伏性的 TB 患者或者健康的对照个体的血液中不如此，在此其过表达/过表现。在图 8E 中也表现了（重叠）。基因 CDKL1(p42)、MICALCL、MBNL3、RHD、ST7(RAY1)、PPR3R1、[360739](PIP5K2A)、AMFR、FLJ22471、CRAT(CAT1)、PLA2G4C、ACOT7(ACT)(ACH1)、RNF182、KLRC3(NKG2E)、HLA-DPB1 在健康的对照个体的血液中低调表达/低表现，然而在潜伏性的 TB 患者的血液中过表达/过表现，并在多数活性的 TB 患者的血液中过表达/过表现（图 8F）。总之，在图 8A 中全部 119 个基因的总体表达谱（为易于理解基因和在临床状态之间的特异性而在图 8B-8F 中进行了划分），所述表达谱将受感染的（活性的 TB 和潜伏性的 TB）患者从未感染的患者（健康的对照）中区分开，并且除此之外，将两组受感染的患者区分开，也即活性的和潜伏性的 TB 患者。通过这种方法，在活性的 TB 患者的血液中过表达的多个基因即使用最严格的统计学筛选所鉴定的相同的基因（示于图 7，表 6），并且许多是 IFN- 可诱导的和/或涉及于胞吞作用的细胞交通和/或脂类代谢。

[0134] 表 6. 发现在潜伏性的和健康的之间，或者在活性的和健康的之间显著差别地表达的基因，其当组合地使用时使用无监管的皮尔逊校正系数聚类算法（119 个基因）区分活性的、健康的和潜伏性的。

[0135]

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基因符号	描述
HMFN0839	肺癌转移相关蛋白
LOC653820	
MID1IP1	MID1 相互作用蛋白 1 (原肠特异性的 G12 类似物 (斑马鱼))
SPTAN1	血影蛋白, α, 非红细胞 1 (α-胞衬蛋白)
NALP12	NLR 家族, 含有热蛋白功能域 12
PSMB8	蛋白酶体 (前体, 巨蛋白因子) 亚单位, β 型, 8 (大的多功能肽酶 7)
RNF182	环指蛋白 182
KCNMB1	钾的高电导率钙激活的通道, 亚家族 M, β 成员 1
	白细胞介素 23, α 亚单位 p19
CDKL1	细胞周期蛋白-依赖的激酶-样 1 (CDC2-相关的激酶)
IL8	白细胞介素 8
NOV	肾母细胞瘤过表达基因
APOL6	载脂蛋白 L, 6
KLRC3	杀伤细胞凝集素-样受体亚家族 C, 成员 3
SOX8	SRY (性别决定区域 Y)-盒 8
B3GALT7	UDP-GlcNAc:βGal β-1,3-N-乙酰葡萄糖胺基转移酶 8
GCH1	GTP 环水解酶 1 (多巴-响应的肌力失常)
IL6R	白细胞介素 6 受体
RASGRP4	RAS 鸟苷酸释放蛋白 4
SGK	血清/糖皮质激素调控的激酶
LOC389541	类似于 CG14977-PA
MICALCL	MICAL C-端样
VAMP3	囊泡关联的膜蛋白 3 (小细胞小泡蛋白)
NPC2	尼曼-皮克病, C2 类型

[0136]

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基因符号	描述
SYNJ2	突触小泡磷酸酶 2
NIN	ninein (GSK3B 相互作用蛋白)
MBNL3	肌肉盲-样 3 (果蝇)
FLJ11259	损伤调控的自噬调节蛋白
NALP12	NLR 家族, 含有热蛋白功能域的 12
LIMK1	
ARSG	芳基硫酸酯酶 G
FLJ33641	染色体 5 开放阅读框 29
PADI4	肽精氨酸脱亚胺酶, 类型 IV
RENBP	肾素结合蛋白
SULF2	硫酸酯酶 2
GSDMDC1	含有 gasdermin 功能域的 1
ST7	致肿瘤性的抑制 7
RBP7	视黄醇结合蛋白 7, 细胞的
HK2	己糖激酶 2
VAMP5	囊泡关联的膜蛋白 5 (肌短蛋白)
GPR177	G 蛋白偶联受体 177
CES1	羧酸酯酶 1 (单核细胞/巨噬细胞丝氨酸酯酶 1)
CD52	CD52 分子
ABCG1	ATP-结合盒,亚家族 G (WHITE), 成员 1
GBP5	鸟苷酸结合蛋白 5
MDM4	Mdm4, 转化的 3T3 细胞双微体 4, p53 结合蛋白(小鼠)
SIGLEC5	唾液酸结合 Ig-样凝集素 5
ARID3A	AT 丰富的活动功能域 3A (BRIGHT-样)
KIAA0179	核糖体 RNA 加工 1 类似物 B (酿酒酵母)
PSD4	含有普列克底物蛋白和 Sec7 功能域的 4
ALOX5AP	花生四烯酸酯 5-脂肪氧合酶-活化蛋白
CSF2RA	集落刺激因子 2 受体, α, 低亲和性的 (粒细胞-巨噬细胞)
MMP9	基质金属肽酶 9 (明胶酶 B, 92kDa 明胶酶, 92kDa 类型 IV 胶原蛋白酶)
PGLYRP1	肽聚糖识别蛋白 1
CYP27A1	细胞色素 P450,家族 27, 亚家族 A, 多肽 1
LMTK2	狐猴酪氨酸激酶 2

[0137]

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基因符号	描述
BRI3	脑蛋白 I3
PILRA	配对的免疫球蛋白样类型 2 受体 α
	锌指蛋白 404
FLJ21127	构造的 1
GBP2	鸟苷酸结合蛋白 2, 干扰素-可诱导的
ST3GAL6	ST3 β-半乳糖苷 α-2,3-唾液酸基转移酶 6
PLAUR	纤溶酶原激活物, 尿激酶受体
NCF4	嗜中性粒细胞胞液因子 4, 40kDa
JAK2	Janus 激酶 2 (蛋白质酪氨酸激酶)
SREBF1	固醇调控元件结合的转录因子 1
SELM	硒蛋白 M
PPP3R1	蛋白质磷酸酶 3 (此前的 2B), 调控亚单位 B, α 同工型
PRKCA	蛋白质激酶 C, α
PLA2G4C	磷脂酶 A2, group IVC (胞液的, 钙离子无关的)
GBP4	鸟苷酸结合蛋白 4
HIP1	舞蹈病蛋白的相互作用蛋白 1
PGM2	葡萄糖磷酸变位酶 2
	KIAA1632
S100P	S100 钙结合蛋白 P
IL27RA	白细胞介素 27 受体, α
IL15	白细胞介素 15
FHIT	脆弱的组氨酸三重基因
FAM84B	具有序列类似性 84 的家族, 成员 B
MCCC1	甲基丁烯酰辅酶 A 羧化酶 1 (α)
ACOT7	酰基辅酶 A 硫酯酶 7
TNFRSF12A	肿瘤坏死因子受体超家族, 成员 12A
SF3A1	剪接因子 3a, 亚单位 1, 120kDa
TNFSF14	肿瘤坏死因子(配体) 超家族, 成员 14
CD300LB	CD300 分子-样家族成员 b
ANPEP	丙胺酰基(膜)氨基肽酶 (氨基肽酶 N, 氨基肽酶 M, 微粒体氨基肽酶, CD13, p150)
FAM7A1	
RHD	Rh 血型, D 抗原

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基因符号	描述
HOM-TES-103	有待证实的蛋白 LOC25900
CCDC41	含有卷曲螺旋功能域的 41
CRNKL1	曲颈前 mRNA 剪接因子-样 1 (果蝇)
NCF1	嗜中性粒细胞胞液的因子 1, (慢性肉芽肿病, 常染色体 1)
UBTD1	含泛素蛋白功能域的 1
FLJ22471	含卷曲螺旋功能域的 92
FAM101B	具有序列类似性 101 的家族, 成员 B
LOC284757	
LOC649377	
CDK5R1	细胞周期蛋白-依赖的激酶 5, 调控亚单位 1 (p35)
	神经母细胞瘤 Cot 25 的全长 cDNA 克隆 CS0DC025YP03 - 按照智人(人类)进行正规化
MBNL3	肌肉盲-样 3 (果蝇)
PSTPIP1	脯氨酸-丝氨酸-苏氨酸磷酸酶相互作用蛋白 1
WARS	色氨酰基-tRNA 合成酶
HLA-DPB1	主要组织相容性复合体, 类型 II, DP β 1
LOC652616	
ACTA2	肌动蛋白, α 2, 平滑肌, 主动脉
IBRDC3	含有 IBR 功能域的 3
PHKG2	磷酸化酶激酶, γ 2 (睾丸)
	磷脂酰肌醇-4-磷酸酯 5-激酶, 类型 II, α
LOC642035	
AMFR	
RGS19	G-蛋白信号转导调节子 19
C22orf5	染色体 22 开放阅读框 5
ATF3	活化转录因子 3
SIPA1L1	信号诱导的增殖关联的 1 样 1
MRPS34	线粒体的核糖体蛋白 S34
ADAL	腺甙脱氨酶-样
NDUFAF1	NADH 脱氢酶 (泛醌) 1 α 亚复合物 (subcomplex), 装配因子 1
CRAT	肉碱乙酰基转移酶

[0139]

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基因符号	描述
STX11	突触融合蛋白 11

[0140] 在活性的和潜伏性的 TB 中不同的和相互的免疫标签通过模块的方法揭示。为了得到关于发病机理的进一步信息，经校准的各个芯片的数据使用刚刚描述的稳定模块分析框架进行进一步分析，所述框架基于预先设定的基因转录物的聚类，所述聚类显示出在宽范围的疾病中共同表达，并通常在功能的水平上表现分子或者细胞的聚类 (Chaussabel 等，2008，Immunity)。

[0141] 由于本分析的目标是得到各组转录标签中所含的基因的功能性信息，所述分析致力于在我们此前的分析中紧密地聚类到一起的患者的亚集合，排除了落单者，其原因在于这样的组更可能显示出涉及到疾病过程的共同路径及过程。

[0142] 选择了九名具有活性 TB 的患者，六名健康的对照以及九名具有潜伏性的 TB 的患者，并将其用于模块分析中。将各个比较分别地进行，从而在一个分析中将九名活性的 TB 患者与六名健康的对照进行比较，随后在另外的分析中将九名潜伏性的患者与同样的六名健康的对照进行比较。对转录物进行筛选，从而排除在所比较的任意组的至少两个个体中任何未检测出来的。随后对患者和健康的对照组进行统计比较（非参数的 Wilcoxon-Mann-Whitney 试验，$P < 0.05$），从而确定在患者组和健康的对照之间有差别地表达的基因。这些有差别地表达的基因随后划分到那些在疾病组中与对照相比上调 / 过表现的那些中，以及在疾病组中与对照相比下调 / 低表现的那些中。这些列表随后通过模块基础在模块上进行分析。在各个模块中有差别地表达的基因或者显著地过表达，或者显著地低表达。为了确保确定，各个模块在表现的方面必须有 > 25% 的总基因变化，并且在特定的方面变化的基因的数目必须 > 10。为了图示全部的转录变化，在活性的 TB 相对健康的对照中，或者潜伏性的 TB 相对健康的对照中，在网格上排列，其各个位置按照它们最初的定义与不同的模块相对应。点强度显示了在所显示方向上有差别地表达的转录物占在该模块中检测到的转录物的总数的比例，而点颜色显示了变化的极性（红色：过表达 / 表现，蓝色：低表达 / 表现）。除此之外，可以将模块坐标与功能的注解相关联，从而便于数据解释 (Chaussabel，Immunity，2008；以及图 9 和 10)。

[0143] 与健康的对照相比，活性的 TB 的模块图谱（图 9，表 7A-P；以及表 8）显示出不同于和健康的对照相比的潜伏性的 TB 的图谱（图 10，表 7A-F；和表 9）。实际上，与健康的对照相比在对两种疾病状态下都显示变化的模块中，这些从活性的 TB 和潜伏性的 TB 独立地产生的模块图谱显示了基因表达 / 表现的相反的样式。与细胞毒素细胞关联的在模块 M2.1 中的基因在活性的 TB 中低表达 / 表现（36% - 表 6F 中列出的基因中，在模块中检测的 50 个中的 18 个基因低表达 / 表现），并且在潜伏性的 TB 中过表达 / 表现（43% - 表 7B 中列出的基因中，在模块中检测的 51 个中的 22 个基因过表达 / 表现）。从另一方面来说，M3.2 和 M3.3 中的多个基因（“炎症”）（列于表 6J 和 6K 的基因）在活性的 TB 患者中过表达 / 表现，然而在潜伏性的 TB 患者中低表达 / 表现（列于表 7E 和 7F 的基因）。类似地，在 M1.5 中的基因（“骨髓系”）在活性的 TB 中过表达 / 表现（列于表 6D 的基因），然而在潜伏性的 TB 中低表达 / 表现（列于表 7A 的基因）。在模块 M2.10 中的基因（所述模块未形成连贯的功能性模块，而是由表面上不同的基因集合组成）在潜伏性的 TB 中低表达 / 表现（列于

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表 7D 中的基因），然而与对照相比在活性的 TB 中不过表达或者低表达 / 表现。这些基因之一是 toll- 样受体接头，TRAM，其是 TLR-4(LPS) 和 TLR-3(dsRNA) 信号转导的下游 (Akira，Nat. Rev. Imm.)。

[0144] 对于表 7A 到 7O，对于活性的 TB 的相对经校正的表达以在活性的患者相对于对照的表达给出。在表 8A 及 8F 中，对于潜伏性的 TB 的相对经校正的表达以在健康对照相对于潜伏性的患者的表达给出。

[0145] 表 7A M1.2PTBv. 对照，在活性的 TB 中过表现的基因。

[0146]

相对的经校正的表达	普通名称	基因符号	描述
	P22_15_PTBvCSelect_09May08_PAL2Ttest_UP_M1.2		
2.447	KX; X1k; XKR1	XK	X 相关的 Kx 血型 (McLeod 综合征)
2.239	CD62; GRMP; PSEL; CD62P; GMP140; PADGEM; FLJ45155	SELP	选择蛋白 P (颗粒膜蛋白 140kDa, 抗原 CD62)
2.161	URG	EGF	表皮生长因子 (β-抗溃疡素)
2.133	JAMC; JAM-C; FLJ14529	JAM3	连接粘合分子 3
2.13	H2B; GL105; H2B.1; H2B/q; H2BFQ; MGC129733; MGC129734	HIST2H2BE	组蛋白簇 2, H2be
1.889	4.1O; P410;	FRMD3	含有 FERM 功能域的 3

[0147]

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相对的经校正的表达	普通名称	基因符号	描述
	EPB41L4O; MGC20553; RP11-439K3.2		
1.875	CKLFSF5; FLJ37521	CMTM5	含有 CKLF-样 MARVEL 跨膜功能域的 5
1.829	ECM; MMRN; GPIa*; EMILIN4	MMRN1	多聚素 1
1.757	PSA; PROS; PS21; PS22; PS23; PS24; PS25; PS 26; Protein S; protein Sa	PROS1	蛋白质 S (α)
1.752	F13A	F13A1	凝固因子 XIII, A1 多肽
1.698	H2B/S; H2BFT; H2BFAiii; MGC131989	HIST1H2BK	组蛋白簇 1, H2bk
1.638		RTN2	
1.59	TMSA; HTM-α; TPM1-α; TPM1-κ	TPM1	原肌球蛋白 1 (α)
1.419		C6orf79	
1.408	BSS; GP1B; CD42B; MGC34595; CD42b-α	GP1BA	糖蛋白 Ib (血小板), α 多肽
1.338	CD61; GP3A; GPIIIa	ITGB3	整合素, β 3 (血小板 糖蛋白 IIIa, 抗原 CD61)
1.183	CMIP; KIAA1694	CMIP	c-Maf-诱导蛋白

[0148] 表 7B M1.3PTBv. 对照，在活性的 TB 中低表达的基因。

[0149]

相对的经校正的表达	普通名称	基因符号	描述
	P22_15_PTBvCSelect_09May08_PAL2Ttest_		

[0150]

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相对的经校正的表达	普通名称	基因符号	描述
	DOWN_M1.3		
0.82	FLJ31738; KIAA1209	PLEKHG1	含有普列克底物蛋白类似物功能域的，家族 G（具有 RhoGef 功能域）成员 1
0.778	SPI-B	SPIB	Spi-B 转录因子（Spi-1/PU.1 相关的）
0.767	EVI9; CTIP1; BCL11A-L; BCL11A-S; FLJ10173; FLJ34997; KIAA1809; BCL11A-XL	BCL11A	B-细胞 CLL/淋巴瘤 11A（锌指蛋白）
0.715	MGC20446	CYBASC3	细胞色素 b，抗坏血酸盐依赖的 3
0.677	NIDD; MGC42530	ZDHHC23	锌指，含有 DHHC 类型的 23
0.629	ESG; ESG1; GRG1	TLE1	分裂 1(E(sp1) 类似物，果蝇）的转运素样增强子
0.612	B29; IGB	CD79B	CD79b 分子，免疫球蛋白关联的 β
0.581	LYB2; CD72b	CD72	CD72 分子
0.559	KIAA0977	COBLL1	COBL 样 1
0.556	BASH; Ly57; SLP65; BLNK-s; SLP-65; MGC111051	BLNK	B 细胞连接物
0.543	TCL1	TCL1A	T 细胞白血病/淋巴瘤 1A
0.518	c-Myc	MYC	v-myc 髓细胞组织增生的病毒性致癌基因类似物（鸟类的）
0.512	BANK; FLJ20706; FLJ34204	BANK1	具有锚蛋白重复 1 的 B-细胞的支架蛋白
0.51	B4; MGC12802	CD19	CD19 分子

[0151]

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相对的经校正的表达	普通名称	基因符号	描述
0.496	FCRH1; IFGP1; IRTA5; RP11-367J7.7; DKFZp667O1421	FCRL1	Fc 受体样 1
0.487	FLJ00058	GNG7	鸟嘌呤核苷酸结合蛋白(G 蛋白), γ 7
0.482	FLJ21562; FLJ43762	C13orf18	染色体 13 开放阅读框 18
0.477	BRDG1; STAP1	BRDG1	BCR 下游信号转导 1
0.471	MGC10442	BLK	B 淋巴球酪氨酸激酶
0.467	R1; JPO2; RAM2; DKFZp762L0311	CDCA7L	细胞分裂周期关联的 7-样
0.445	ORP10; OSBP9; FLJ20363	OSBPL10	氧化固醇结合蛋白样 10
0.397	8HS20; N27C7-2	VPREB3	前 B 淋巴细胞基因 3
0.361	LAF4; MLLT2 样	AFF3	AF4/FMR2 家族, 成员 3
0.334	FCRL; FREB; FCRLX; FCRLb; FCRLd; FCRLe; FCRLM1; FCRLc1; FCRLc2; MGC4595; RP11-474I16.5	FCRLM1	Fc 受体样 A

[0152] 表 7C M1.4PTBv. 对照,在活性的 TB 中低表现的基因

[0153]

相对的经校正的表达	普通名称	基因符号	描述
	P22_15_PTBvCSelect_09May08_PAL2Ttest_DOWN_M1.4		
0.907	FLJ12298; ZKSCAN14	ZNF394	锌指蛋白 394
0.835	JMY; FLJ37870;	JMY	联接介导及调控蛋白

[0154]

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相对的经校正的表达	普通名称	基因符号	描述
	MGC163496		
0.825	C1; C2; HNRNP; SNRPC; hnRNPC; MGC104306; MGC105117; MGC117353; MGC131677	HNRPC	异种的核糖核蛋白 C (C1/C2)
0.78	SON3; BASS1; DBP-5; NREBP; C21orf50; FLJ21099; FLJ33914; KIAA1019	SON	SON DNA 结合蛋白
0.77	HMGE; FLJ25609	GRPEL1	GrpE 样 1, 线粒体的(E. coli)
0.747	HEPP; FLJ20764; MGC19517	CDCA4	细胞分裂周期关联 4
0.723	RITA; ZNF361; ZNF463; DKFZp686L0787	ZNF331	锌指蛋白 331
0.698	FLJ12670; FLJ20436	C12orf41	染色体 12 开放阅读框 41
0.698	DRBF; MMP4; MPP4; NF90; NFAR; TCP80; DRBP76; NFAR-1; MPHOSPH4; NF-AT-90	ILF3	白细胞介素增强子结合因子 3, 90kDa
0.689	TIMAP; ANKRD4; KIAA0823	PPP1R16B	蛋白质磷酸酶 1,调控(抑制剂)亚单位 16B
0.678	PRP21; PRPF21; SAP114; SF3A120	SF3A1	剪接因子 3a, 亚单位 1, 120kDa
0.667	SDS; SWDS; CGI-97; FLJ10917	SBDS	Shwachman-Bodian-Diamond 综合征
0.665	BL11; HB15	CD83	CD83 分子
0.645	NOT; RNR1; HZF-3;	NR4A2	核受体亚家族 4, 组 A, 成员 2

[0155]

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相对的经校正的表达	普通名称	基因符号	描述
	NURR1; TINUR		
0.62	H1RNA	RNASEH1	核糖核酸酶 H1

[0156] 表 7D M1.5PTBv. 对照，在活性的 TB 中过表现的基因。

[0157]

相对的经校正的表达	普通名称	基因符号	描述
	P22_15_PTBvCSelect_09May08_PAL2Ttest_UP_M1.5		
2.384	VHR	DUSP3	双特异性磷酸酶 3 (痘苗病毒磷酸酶 VH1-相关的)
2.139	4.1B; DAL1; DAL-1; FLJ37633; KIAA0987	EPB41L3	红细胞膜蛋白带 4.1 样 3
2.014	HXK3; HKIII	HK3	己糖激酶 3 (白细胞)
1.972	HL14; MGC75071	LGALS2	凝集素，结合半乳糖苷酶，可溶的，2
1.844	KYNU	KYNU	犬尿氨酸酶 (L-犬尿氨酸水解酶)
1.618	BLVR; BVRA	BLVRA	胆绿素还原酶 A
1.594	RP35; SEMB; SEMAB; CORD10; FLJ12287; RP11-54H19.2	SEMA4A	sema 功能域，免疫球蛋白功能域 (Ig)，跨膜功能域 (TM) 以及短的细胞质的功能域，(脑信号蛋白) 4A
1.535		GRN	
1.531	G6S; MGC21274	GNS	葡萄糖胺 (N-乙酰基)-6-硫酸酯酶 (Sanfilippo 疾病 IIID)
1.524	FOAP-10; EMILIN-2; FLJ33200	EMILIN2	弹性蛋白微纤维界面蛋白 2

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[0158]

相对的经校正的表达	普通名称	基因符号	描述
1.507	cent-b; HSA272195	CENTA2	矢车菊苷, α 2
1.449	APPS; CPSB	CTSB	组织蛋白酶 B
1.438	ASGPR; CLEC4H1; Hs.12056	ASGR1	无唾液酸糖蛋白受体 1
1.433	CD32; FCG2; FcGR; CD32A; CDw32; FCGR2; IGFR2; FCGR2A1; MGC23887; MGC30032	FCGR2A	IgG 的 Fc 片段, 低亲和性 IIa, 受体 (CD32)
1.425	TIL4; CD282	TLR2	toll-样受体 2
1.424	PI; A1A; AAT; PI1; A1AT; MGC9222; PRO2275; MGC23330	SERPINA1	丝氨酸蛋白酶抑制蛋白肽酶抑制剂, 进化枝 A (α-1 抗蛋白酶, 抗胰蛋白酶), 成员 1
1.413	TEM7R; FLJ14623	PLXDC2	含有丛蛋白功能域的 2
1.41	CD14	CD14	CD14 分子
1.398	Rab22B	RAB31	RAB31, 成员 RAS 致癌基因家族
1.386	FEX1; FEEL-1; FELE-1; STAB-1; CLEVER-1; KIAA0246	STAB1	稳定素 1
1.352	MYD88	MYD88	骨髓分化初级反应基因(88)
1.349	MLN70; S100C	S100A11	S100 钙结合蛋白 A11
1.347	FLJ22662	FLJ22662	有待证实的蛋白质 FLJ22662
1.346	CLN2; GIG1; LPIC; TPP I; MGC21297	TPP1	三肽基肽酶 I
1.251	p75; TBPII; TNFBR; TNFR2; CD120b;	TNFRSF1B	肿瘤坏死因子受体超家族, 成员 1B

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相对的经校正的表达	普通名称	基因符号	描述
	TNFR80; TNF-R75; p75TNFR; TNF-R-II		
1.239	JTK9	HCK	造血细胞激酶
1.172	IBA1; AIF-1; IRT-1	AIF1	异种移植物炎症因子 1

[0160] 表 7E M1.8PTBv. 对照，在活性的 TB 中低表现的基因。

[0161]

相对的经校正的表达	普通名称	基因符号	描述
	P22_15_PTBvCSelect_09May08_PAL2Ttest_DOWN_M1.8		
0.878	DBP2; PRP8; DDX16; PRO2014	DHX16	DEAH (Asp-Glu-Ala-His)盒多肽 16
0.858	AN11; HAN11	WDR68	WD 重复功能域 68
0.843	NDR; NDR1	STK38	丝氨酸/苏氨酸激酶 38
0.821	FLJ20097; FLJ23581; KIAA1861	FLJ20097	含有卷曲螺旋功能域的 132
0.814	FLJ42526; FLJ45813; MGC71764	RSBN1L	圆形精子细胞碱性蛋白 1 样
0.809	C9orf55; C9orf55B; FLJ20686; bA513M16.3; DKFZp686I09113	DENND4C	含有 DENN/MADD 功能域的 4C
0.808	SON3; BASS1; DBP-5; NREBP; C21orf50; FLJ21099; FLJ33914; KIAA1019	SON	SON DNA 结合蛋白
0.807	p150; VPS15; MGC102700	PIK3R4	磷酸肌醇-3-激酶，调控亚单位 4, p150

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[0162]

相对的经校正的表达	普通名称	基因符号	描述
0.8	4E-T; Clast4; FLJ21601; FLJ26551	EIF4ENIF1	真核翻译起始因子 4E 核输入因子 1
0.798	TAF2D; TAFII100	TAF5	TAF5 RNA 聚合酶 II, TATA 盒结合蛋白(TBP)-关联因子, 100kDa
0.793	DBR1	DBR1	脱支酶类似物 1 (酿酒酵母)
0.785	SMAP; p120; SMAP2	BRD8	含有溴代功能域的 8
0.785		CASP2	
0.772	TRF2; TRBF2	TERF2	端粒重复序列结合因子 2
0.772	hNUP133; FLJ10814; MGC21133	NUP133	核孔蛋白 133kDa
0.762	MGC4268; FLJ38552	MGC4268	AMME 染色体区域基因 1 样
0.761	PUMH2; PUML2; FLJ36528; KIAA0235; MGC138251; MGC138253	PUM2	pumilio 类似物 2 (果蝇)
0.751	BYE1; DIO1; DATF1; DIDO2; DIDO3; DIO-1; FLJ11265; KIAA0333; MGC16140; C20orf158; dJ885L7.8; DKFZp434P1115	DIDO1	诱导死亡泯灭因子 1
0.738	KOX5; ZNF13	ZNF45	锌指蛋白 45
0.727	FLJ20558	FLJ20558	染色体 2 开放阅读框 42
0.713	FLJ32343	CWF19L2	CWF19 样 2, 细胞周期控制 (裂殖酵母)
0.709	MGC16770	RAB22A	RAB22A, 成员 RAS 致癌基因家族

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相对的经校正的表达	普通名称	基因符号	描述
0.708	FLJ14431	CBR 4	羰基还原酶 4
0.704	AASDH; NRPS998; NRPS1098	AASDH	2-氨基己二基 6-半醛脱氢酶
0.698	ZSCAN11	ZNF232	锌指蛋白 232
0.692	NudCL; KIAA1068	NUDCD3	含有 NudC 功能域的 3
0.691	CCA1; MtCCA; CGI-47	TRNT1	tRNA 核苷酸转移酶, CCA-加成, 1
0.689	RBM30; RBM4L; ZCRB3B; ZCCHC15; MGC10871	RBM4B	RNA 结合基序蛋白质 4B
0.683	CLF; CRN; HCRN; SYF3; MSTP021	CRNKL1	曲颈前 mRNA 剪接因子样 1 (果蝇)
0.676	ZBU1; HLTF1; RNF80; HIP116; SNF2L3; HIP116A; SMARCA3	SMARCA 3	螺旋酶样转录因子
0.666	SWAN; KIAA0765; HRIHFB2091	RBM12	RNA 结合基序蛋白质 12
0.658	FLJ10287; FLJ11219	CCDC76	含有卷曲螺旋功能域的 76
0.654	INT5; KIAA1698	KIAA169 8	整合复合物亚单位 5
0.652	IAN7; hIAN7; MGC27027	GIMAP7	GTP 酶, IMAP 家族成员 7
0.651	TTC20; DKFZP586B0923	KIAA127 9	KIAA1279
0.65	RAL; MGC48949	RALA	v-ral 类人猿白血病病毒性致癌基因类似物 A (ras 相关的)
0.639	MPRB; LMPB1; C6orf33; FLJ32521; FLJ46206	PAQR8	孕酮和 adipoQ 受体家族成员 VIII

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相对的经校正的表达	普通名称	基因符号	描述
0.634	FLJ11171	FLJ11171	待证实的蛋白质 FLJ11171
0.613	LCF; IL-16; prIL-16; FLJ16806; FLJ42735; FLJ44234; HsT19289	IL16	白细胞介素 16 (淋巴细胞化学引诱物因子)
0.611	FLJ33226; 1190004M21Rik	PYGO2	pygopus 类似物 2 (果蝇)
0.577	GLC1G; UTP21; TAWDRP; TA-WDRP; DKFZp686I1650	WDR36	WD 重复功能域 36
0.574	FLJ20287; bA208F1.2; RP11-208F1.2	TEX10	睾丸表达的 10
0.568	KIAA1982	ZNF721	锌指蛋白 721
0.55	FLJ22457; RP5-1180E21.2	DENND2D	含有 DENN/MADD 功能域的 2D
0.545	ozrf1; ZFP260	ZFP260	锌指蛋白 260
0.491	GLS1; FLJ10358; KIAA0838; DKFZp686O15119	GLS	谷氨酰胺酶

[0165] 表 7F M2. 1PTBv. 对照，在活性的 TB 中低表现的基因 .

[0166]

相对的经校正的表达	普通名称	基因符号	描述
	P22_15_PTBvCSelect_09May08_PAL2Ttest_DOWN_M2.01		
0.712	PTPMEG; PTPMEG1	PTPN4	蛋白质酪氨酸磷酸酶，非受体类型 4 (巨核细胞)
0.665	FLJ34563; MGC35163	SAMD3	含有 sterile α 基序功能域的 3

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[0167]

0.643	STAT4	STAT4	信号转导器以及转录4的活化剂
0.638	DIL1; DIL-1; Mindin; M-spondin	SPON2	spondin 2, 细胞外基质蛋白
0.631	SLP2; SGA72M; CHR11SYT; KIAA1597; MGC102768	SYTL2	突触结合蛋白样 2
0.628	DORZ1; DKFZP564O243	ABHD14A	含有 Ab 水解酶功能域的 14A
0.615	LPAP; CD45-AP; MGC138602; MGC138603	PTPRCAP	蛋白质酪氨酸磷酸酶, 受体类型, C-关联蛋白
0.595	PKCL; PKC-L; PRKCL; MGC5363; MGC26269; nPKC-eta	PRKCH	蛋白质激酶 C, η
0.581	MGC33870; MGC74858	NCALD	神经钙蛋白 δ
0.566	T11; SRBC	CD2	CD2 分子
0.554	KLR; CD314; NKG2D; NKG2-D; D12S2489E	KLRK1	杀伤细胞凝集素-样受体亚家族 K, 成员 1
0.546	LAX; FLJ20340	LAX1	淋巴细胞跨膜接头 1
0.529	CD122; P70-75	IL2RB	白细胞介素 2 受体, β
0.515	FEZ1	FEZ1	簇生和延长蛋白质 ζ1 (zygin I)
0.509	CHK; CTK; HYL; Lsk; HYLTK; HHYLTK; MGC1708; MGC2101; DKFZp434N1212	MATK	巨核细胞关联的酪氨酸激酶
0.468	CLIC3	CLIC3	氯化物胞内通道 3
0.439	1C7; CD337; LY117; NKp30	NCR3	天然细胞毒性触发受体 3
0.39	TRYP2	GZMK	粒酶 K (粒酶 3; 类胰蛋白酶

[0168]

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			II)

[0169] 表 7G M2.4PTBv. 对照，在活性的 TB 中低表现的基因。

[0170]

相对的经校正的表达	普通名称	基因符号	描述
	P22_15_PTBvCSelect_09May08_PAL2Ttest_DOWN_M2.04		
0.858	ATPO; OSCP	ATP5O	ATP 合成酶，H+ 转运，线粒体的 F1 复合物，O 亚单位 (寡霉素敏感授予蛋白质)
0.831	M9; eIF3k; ARG134; PTD001; HSPC029; MSTP001; PLAC-24; PRO1474	EIF3S12	真核翻译起始因子 3，亚单位 12
0.822	RPL8	RPL8	核糖体蛋白质 L8
0.811	EF2; EEF-2	EEF2	真核翻译延长因子 2
0.804	RPB9; hRPB14.5	POLR2I	聚合酶 (RNA) II (DNA 定向的) 多肽 I，14.5kDa
0.801	RP8; ZMYND7; MGC12347	PDCD2	程序性细胞死亡 2
0.788	ARI2; TRIAD1; FLJ10938; FLJ33921	ARIH2	ariadne 类似物 2 (果蝇)
0.776	Erv46; CGI-54; PRO0989; C20orf47; NY-BR-84; SDBCAG84; dJ477O4.2	ERGIC3	ERGIC 和高尔基体 3
0.771	ART-27	UXT	无所不在地表达的转录物
0.769	H12.3; HLC-7; PIG21; RACK1; Gnb2-rs1	GNB2L1	鸟嘌呤核苷酸结合蛋白(G 蛋白)，β 多肽 2 样 1
0.766	eIF3h; eIF3-p40; MGC102958; eIF3-γ	EIF3S3	真核翻译起始因子 3，亚单位 3 γ，40kDa

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[0171]

相对的经校正的表达	普通名称	基因符号	描述
0.759	HCA56	LGTN	连接素
0.758	2PP2A; IGAAD; I2PP2A; PHAPII; TAF-IBETA	SET	SET 易位 (骨髓白血病关联的)
0.752	ANG2	C11orf2	染色体 11 开放阅读框 2
0.74	C6.1B	MTCP1	成熟的 T 细胞增殖 1
0.736	LCP; HCLP-1	KLHDC2	含有 kelch 功能域的 2
0.722	DKFZP566B023	RPL36	核糖体蛋白质 L36
0.712	KOX30	ZNF32	锌指蛋白 32
0.71	AMP; MGC125856; MGC125857; MGC129961; DKFZp686D13177	APRT	腺嘌呤磷酸核糖转移酶
0.694	GDH; MGC149525; MGC149526; λ-CRY	CRYL1	晶状体蛋白, λ 1
0.689	FLJ27451; MGC102930	RPS20	核糖体蛋白质 S20
0.686	INT6; eIF3e; EIF3-P48; eIF3-p46	EIF3S6	真核翻译起始因子 3, 亚单位 6 48kDa
0.68	LK4; hCERK; FLJ21430; FLJ23239; KIAA1646; MGC131878; dA59H18.2; dA59H18.3; DKFZp434E0211	CERK	神经酰胺激酶
0.675	HINT; PKCI-1; PRKCNH1	HINT1	三聚组氨酸核苷酸结合蛋白 1
0.675	NHP2; NHP2P	NOLA2	核仁蛋白家族 A, 成员 2 (H/ACA 小核仁 RNPs)
0.668	AMP; MGC125856;	APRT	腺嘌呤磷酸核糖转移酶

[0172]

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相对的经校正的表达	普通名称	基因符号	描述
	MGC125857; MGC129961; DKFZp686D13177		
0.667	TOM7	TOMM7	外围线粒体膜 7 类似物的移位酶 (酵母)
0.655	SIVA; CD27BP; Siva-1; Siva-2	SIVA	SIVA1, 凋亡诱导因子
0.646	PBP; HCNP; PEBP; RKIP	PEBP1	磷脂酰乙醇胺结合蛋白 1
0.628	PRP9; PRPF9; SAP61; SF3a60	SF3A3	剪接因子 3a, 亚单位 3, 60kDa
0.62	FLJ12525; dJ475B7.2; RP3-475B7.2	LAS1L	LAS1 样 (酿酒酵母)
0.593	EC45; RPL10; RPLY10; RPYL10; FLJ26304; MGC88603	RPL15	核糖体蛋白质 L15
0.567	HNRNP; JKTBP; JKTBP2; laAUF1	HNRPDL	异种的核核糖蛋白 D 样
0.562	SMD2; SNRPD1	SNRPD2	小核核糖蛋白 D2 多肽 16.5kDa
0.549		PPIA	
0.527	LOC130074; MGC87527	LOC130074	p20
0.524	RDGBB; RDGBB1; RDGB-BETA	PITPNC1	磷脂酰肌醇转移蛋白, 细胞质的 1
0.5	HEI10; C14orf18	CCNB1IP1	细胞周期蛋白 B1 相互作用蛋白 1
0.492	EAP; HBP15; HBP15/L22	RPL22	核糖体蛋白质 L22

[0173] 表 7H M2.8PTBv. 对照, 在活性的 TB 中低表现的基因。

[0174]

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相对的经校正的表达	普通名称	基因符号	描述
	P22_15_PTBvCSelect_09May08_PAL2Ttest_DOWN_M2.08		
0.871	KPL1; PHR1; PHRET1	PLEKHB1	含有普列克底物蛋白类似物功能域的,家族 B (evectins) 成员 1
0.816	MGC132014	INPP4B	纤维醇多磷酸酯-4-磷酸酯酶, 类型 II, 105kDa
0.732	SEP2; SEPT2; KIAA0128; MGC16619; MGC20339; RP5-876A24.2	6-Sep	隔蛋白 6
0.711	GIL	AQP3	水通道蛋白 3 (Gill 血型)
0.691	FLJ36386	LZTFL1	亮氨酸拉链转录因子样 1
0.67	p52; p75; PAIP; DFS70; LEDGF; PSIP2; MGC74712	PSIP1	PC4 和 SFRS1 相互作用蛋白 1
0.669	GRG; ESP1; GRG5; TLE5; AES-1; AES-2	AES	分裂的氨基端增强子
0.668	p33; TNFC; TNFSF3	LTB	淋巴毒素 β (TNF 超家族, 成员 3)
0.646	KIAA0521; MGC15913	ARHGEF18	rho/rac 鸟嘌呤核苷酸交换因子(GEF) 18
0.634	TEM3; TEM7; FLJ36270; FLJ45632; DKFZp686F0937	PLXDC1	含有丛蛋白功能域的 1
0.626	HPIP	PBXIP1	pre-B-细胞白血病同源框相互作用蛋白 1
0.621	KIAA0495; MGC138189	KIAA0495	KIAA0495
0.615	KUP; ZNF46	ZBTB25	含有锌指和 BTB 功能域的 25

[0175]

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相对的经校正的表达	普通名称	基因符号	描述
0.61	FLJ20729; FLJ20760; NY-BR-75; MGC131963	C1orf181	染色体 1 开放阅读框 181
0.609	AAG6; PKCA; PRKACA; MGC129900; MGC129901; PKC-α	PRKCA	蛋白质激酶 C, α
0.604	CGI-25	NOSIP	一氧化氮合成酶相互作用蛋白
0.602	FLJ20152; FLJ22155; FLJ22179	FLJ20152	具有序列相似性 134 的家族, 成员 B
0.599	FRA3B; AP3Aase	FHIT	脆弱的三聚组氨酸基因
0.596	WDR74	WDR74	WD 重复功能域 74; 同义词: FLJ10439, FLJ21730; 智人 WD 重复功能域 74 (WDR74), mRNA.
0.595	E25A; BRICD2A	ITM2A	内在的膜蛋白 2A
0.587	HPF2	ZNF84	锌指蛋白 84
0.58	SEK; HEK8; TYRO1	EPHA4	EPH 受体 A4
0.578	SID1; SID-1; FLJ20174; B830021E24Rik	SIDT1	SID1 跨膜家族, 成员 1
0.557	LTBP2; LTBP-3; pp6425; FLJ33431; FLJ39893; FLJ42533; FLJ44138; DKFZP586M2123	LTBP3	潜伏性的转化生长因子β结合蛋白 3
0.556	V; RASGRP; hRasGRP1; MGC129998; MGC129999; CALDAG-GEFI;	RASGRP1	RAS 乌苷酸释放蛋白 1 (钙和 DAG 调节的)

[0176]

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相对的经校正的表达	普通名称	基因符号	描述
	CALDAG-GEFII		
0.546	TTF; ARHH	RHOH	ras 类似物基因家族, 成员 H
0.545	LAT3; LAT-2; y+LAT-2; KIAA0245; DKFZp686K15246	SLC7A6	溶质载体家族 7 (阳离子氨基酸转运物, y+ 系统), 成员 6
0.541	TP120	CD6	CD6 分子
0.537	MGC29816	CHMP7	CHMP 家族, 成员 7
0.53	DAGK; DAGK1; MGC12821; MGC42356; DGK-α	DGKA	二酰甘油激酶, α 80kDa
0.523	hly9; mLY9; CD229; SLAMF3	LY9	淋巴细胞抗原 9
0.52	EMT; LYK; PSCTK2; MGC126257; MGC126258	ITK	IL2-可诱导的 T 细胞激酶
0.519	TACTILE; MGC22596; DKFZp667E2122	CD96	CD96 分子
0.518	SEP2; SEPT2; KIAA0128; MGC16619; MGC20339; RP5-876A24.2	6-Sep	隔蛋白 6
0.501	SCAP1; SKAP55	SCAP1	src 激酶关联的磷蛋白质 1
0.49	FLJ12884; MGC130014; MGC130015	C10orf38	染色体 10 开放阅读框 38
0.488	T1; LEU1	CD5	CD5 分子
0.487	MAL	MAL	mal, T 细胞分化蛋白
0.484	SATB1	SATB1	SATB 同源框 1
0.48	LDH-H; TRG-5	LDHB	乳酸脱氢酶 B

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相对的经校正的表达	普通名称	基因符号	描述
0.473	Ray; FLJ39121; DKFZP586F1318	SH3YL1	含有 SH3 功能域的, Ysc84 样 1 (酿酒酵母)
0.466	P19; SGRF; IL-23; IL-23A; IL23P19; MGC79388	IL23A	白细胞介素 23, α 亚单位 p19
0.465	KE6; FABG; HKE6; FABGL; RING2; H2-KE6; D6S2245E; dJ1033B10.9	HSD17B8	羟基固醇 (17-β) 脱氢酶 8
0.456	ARH; ARH1; ARH2; FHCB1; FHCB2; MGC34705; DKFZp586D0624	LDLRAP1	低密度脂蛋白质受体接头蛋白质 1
0.453	MGC45416; DKFZp686C03164	OCIAD2	含有 OCIA 功能域的 2
0.451	CD172g; SIRPB2; SIRP-B2; bA77C3.1; SIRPγ	SIRPB2	信号调控蛋白质 γ
0.435	GP40; TP41; Tp40; LEU-9	CD7	CD7 分子
0.427	MGC15763	MGC15763	含有氧化还原酶 NAD-结合功能域的 1
0.41	AS160; DKFZp779C0666	TBC1D4	TBC1 功能域家族, 成员 4
0.404	HMIC; MAN1C; MAN1A3; pp6318	MAN1C1	甘露糖苷酶, α, 1C 类, 成员 1
0.401	Tp44; MGC138290	CD28	CD28 分子
0.394	FLJ12586	ZNF329	锌指蛋白 329
0.39	TCF-1; MGC47735	TCF7	转录因子 7 (T 细胞特异的, HMG-盒)
0.385	ABLIM; LIMAB1; LIMATIN;	ABLIM1	肌动蛋白结合 LIM 蛋白质 1

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相对的经校正的表达	普通名称	基因符号	描述
	MGC1224; FLJ14564; KIAA0059; DKFZp781D0148		
0.383	NSE2; BCMP101	FAM84B	具有序列类似性 84 的家族，成员 B
0.377	TOSO	FAIM3	Fas 凋亡抑制分子 3
0.371	EEIG1; C9orf132; MGC50853; bA203J24.7	C9orf132	具有序列类似性 102 的家族，成员 A
0.36	RIT1; CTIP2; CTIP-2; hRIT1-α	BCL11B	B-细胞 CLL/淋巴瘤 11B (锌指蛋白)
0.33	CLP24; FLJ20898; MGC111564	C16orf30	染色体 16 开放阅读框 30
0.315	TCF1ALPHA; DKFZp586H0919	LEF1	淋巴增强子-结合因子 1
0.29	BLR2; EBI1; CD197; CDw197; CMKBR7	CCR7	趋化因子 (C-C 基序) 受体 7
0.244	STK37; PASKIN; KIAA0135; DKFZP434O051; DKFZp686P2031	PASK	含有 PAS 功能域的丝氨酸/苏氨酸激酶
0.205	NRP2	NELL2	NEL 样 2 (鸡)

[0179] 表 71 M3. 1PTBv. 对照，在活性 TB 中过表现的基因。

[0180]

相对的经校正的表达	普通名称	基因符号	描述
	P22_15_PTBvCSelect_09May08_PAL2Ttest_UP_M3.1		
17.93	MGC22805	ANKRD22	锚蛋白重复功能域 22

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[0181]

相对的经校正的表达	普通名称	基因符号	描述
14.86	C1IN; C1NH; HAE1; HAE2; C1INH	SERPING1	丝氨酸蛋白酶抑制蛋白肽酶抑制剂, 进化枝 G (C1 抑制剂), 成员 1, (血管性水肿, 遗传的)
9.425	cig5; vig1; 2510004L01Rik	RSAD2	含有自由基 S-腺苷甲硫氨酸功能域的 2
8.938	BRESI1; MGC29634	EPSTI1	上皮基质相互作用 1 (胸)
8.226	GS3686; C1orf29	IFI44L	干扰素诱导蛋白 44 样
7.566	GBP1	GBP1	鸟苷酸结合蛋白 1, 干扰素-可诱导的, 67kDa
5.677	p44; MTAP44	IFI44	干扰素诱导蛋白 44
4.701	LAP; PEPS; LAPEP	LAP3	亮氨酸氨基肽酶 3
4.401	IRG2; IFI60; IFIT4; ISG60; RIG-G; CIG-49; GARG-49	IFIT3	具有三十四肽重复 3 的干扰素诱导蛋白
4.091	OIAS; IFI-4; OIASI	OAS1	2',5'- 寡腺苷酸合成酶 1, 40/46kDa
3.947	p100; MGC133260	OAS3	2'-5'- 寡腺苷酸合成酶 3, 100kDa
3.944	G1P2; UCRP; IFI15	G1P2	ISG15 泛素样修饰基因
3.915	UEF1; DRIF2; C7orf6; FLJ39885; KIAA2005	SAMD9L	含有 sterile α 基序功能域的 9 样
3.909	MMTRA1B	PLSCR1	磷脂爬行酶 1
3.792	XAF1; BIRC4BP; HSXIAPAF1	BIRC4BP	XIAP 关联因子-1
3.731	RIGE; SCA2; RIG-E; SCA-2; TSA-1	LY6E	淋巴细胞抗原 6 复合物, 基因座 E
3.726	C7; IFI10; INP10; IP-10; crg-2; mob-1; SCYB10; gIP-10	CXCL10	趋化因子 (C-X-C 基序) 配体 10

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相对的经校正的表达	普通名称	基因符号	描述
3.668	FBG2; FBS2; FBX6; Fbx6b	FBXO6	F-盒蛋白质 6
3.652	RNF94; STAF50; GPSTAF50	TRIM22	含三联基序 22
3.619	LOC129607	LOC129607	待证实的蛋白质 LOC129607
3.419	ISGF-3; STAT91; DKFZp686B04100	STAT1	信号转导器和转录活化剂 1, 91kDa
3.398	TRIP14; p59OASL	OASL	2'-5'-寡腺苷酸合成酶样
3.284	IFP35; FLJ21753	IFI35	干扰素诱导蛋白 35
3.154	LOC26010; DNAPTP6; DKFZp564A2416	DNAPTP6	病毒性 DNA 聚合酶-转移活性的蛋白质 6
3.076	BAL; BAL1; FLJ26637; FLJ41418; MGC:7868; DKFZp666B0810; DKFZp686M15238	PARP9	聚(ADP-核糖) 聚合酶家族, 成员 9
3.032	BAL2; KIAA1268	PARP14	聚(ADP-核糖) 聚合酶家族, 成员 14
2.977	RIG-B; UBCH8; MGC40331	UBE2L6	泛素结合酶 E2L 6
2.839	APT1; PSF1; ABC17; ABCB2; RING4; TAP1N; D6S114E; FLJ26666; FLJ41500; TAP1*0102N	TAP1	转运子 1, ATP-结合盒,亚家族 B (MDR/TAP)
2.814	MX; MxA; IFI78; IFI-78K	MX1	黏病毒 (流感病毒) 抵抗 1, 干扰素-可诱导的蛋白 p78 (小鼠)
2.632		IRF7	
2.511	GCH; DYT5;	GCH1	GTP 环水解酶 1 (多巴-响应

[0183]

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相对的经校正的表达	普通名称	基因符号	描述
	GTPCH1; GTP-CH-1		的肌力失常)
2.434	9-27; CD225; IFI17; LEU13	IFITM1	干扰素诱导的跨膜蛋白 1 (9-27)
2.415	G10P2; IFI54; ISG54; cig42; IFI-54; GARG-39; ISG-54K	IFIT2	具有三十四肽重复2的干扰素诱导蛋白
2.414	Hlcd; MDA5; MDA-5; IDDM19; MGC133047	IFIH1	螺旋酶 C 功能域 1 诱导的干扰素
2.378	P113; ISGF-3; STAT113; MGC59816	STAT2	信号转导器及转录 2 的活化剂, 113kDa
2.321	TL2; APO2L; CD253; TRAIL; Apo-2L	TNFSF10	肿瘤坏死因子 (配体) 超家族, 成员 10
2.32	TEL2; TELB; TEL-2	ETV7	ets 基因变体 7 (TEL2 致癌基因)
2.214	OIAS; IFI-4; OIASI	OAS1	2',5'- 寡腺苷酸合成酶 1, 40/46kDa
2.206	APT2; PSF2; ABC18; ABCB3; RING11; D6S217E	TAP2	转运子 2, ATP-结合盒,亚家族 B (MDR/TAP)
2.134	MGC78578	OAS2	2'-5'- 寡腺苷酸合成酶 2, 69/71kDa
2	VRK2	VRK2	痘苗相关的激酶 2
1.975	PN-I; PSN1; UMPH; UMPH1; P5'N-1; cN-III; MGC27337; MGC87109; MGC87828	NT5C3	5'-核苷酸酶, 胞液的 III
1.895	RNF88; TRIM5α	TRIM5	含三联基序 5
1.89	CGI-34; PNAS-2; C9orf83; HSPC177; SNF7DC2	CHMP5	染色质修饰蛋白 5

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相对的经校正的表达	普通名称	基因符号	描述
1.863	ZC3H1; PARP-12; ZC3HDC1; FLJ22693	PARP12	聚(ADP-核糖)聚合酶 家族, 成员 12
1.845	PKR; PRKR; EIF2AK1; MGC126524	EIF2AK2	真核翻译起始因子 2-α 激酶 2
1.842	90K; MAC-2-BP	LGALS3BP	凝集素, 半乳糖苷酶结合的, 可溶的, 3 结合蛋白
1.807	RNF88; TRIM5α	TRIM5	含三联基序的 5
1.743	C15; onzin	PLAC8	胎盘特异的 8
1.732	p48; IRF9; IRF-9; ISGF3	ISGF3G	干扰素刺激的转录因子 3, γ 48kDa
1.713	CD317	BST2	骨髓基质细胞抗原 2
1.665	ESNA1; ERAP140; FLJ45605; MGC88425; Nbla00052; Nbla10993; dJ187J11.3	NCOA7	核受体共活化剂 7
1.649	FLJ39275; MGC131926	ZNFX1	锌指, 含有 NFX1 型的 1
1.628	VODI; IFI41; IFI75; FLJ22835	SP110	SP110 核体蛋白
1.627	EFP; Z147; RNF147; ZNF147	TRIM25	含三联基序的 25
1.523	NMI	NMI	N-myc (和 STAT)相互作用剂
1.505	TRAP; KIAA1529; PCTAIRE2BP; RP11-508D10.1	TDRD7	含有 tudor 功能域的 7
1.499	DSH; G1P1; IFI4; p136; ADAR1; DRADA; DSRAD; IFI-4; K88dsRBP	ADAR	腺甙脱氨酶, RNA-特异的

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相对的经校正的表达	普通名称	基因符号	描述
1.494	C1GALT; T-合成酶	C1GALT1	核心 1 合成酶, 糖蛋白-N-乙酰氨基半乳糖 3-β-半乳糖基转移酶, 1
1.478		PHF11	
1.461	SCOTIN	SCOTIN	scotin
1.433	FLJ00340; FLJ34579; DKFZp686E07254	SP100	SP100 核抗原
1.415	FLJ45064	AGRN	集聚蛋白
1.351	NFTC; OEF1; OEF2; C7orf5; FLJ20073; KIAA2004	SAMD9	含有 sterile α 基序功能域的 9
1.26	MEL; RAB8	RAB8A	RAB8A, 成员 RAS 致癌基因家族
1.215	6-16; G1P3; FAM14C; IFI616; IFI-6-16	G1P3	干扰素, α-可诱导的蛋白 6

[0186] 表 7J M3.2PTBv. 对照, 在活性的 TB 中过表现的基因。

[0187]

相对的经校正的表达	普通名称	基因符号	描述
	P22_15_PTBvCSelect_09May08_PAL2Ttest_UP_M3.2		
2.767	MGC20461	OSM	制瘤素 M
2.202	FHL4; HLH4; HPLH4	STX11	突触融合蛋白 11
2.136	LPCAT2; FLJ20481; LysoPAFAT; DKFZp686H22112	AYTL1	酰基转移酶样 1
1.987	UP; UPP; UPASE; UDRPASE	UPP1	尿苷磷酸化酶 1

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相对的经校正的表达	普通名称	基因符号	描述
1.969	IL-1; IL1F2; IL1-BETA	IL1B	白细胞介素 1, β
1.886	SAT; DC21; KFSD; SSAT; SSAT-1	SAT	亚精胺/精胺 N1-乙酰基转移酶 1
1.862	PFK2; IPFK2	PFKFB3	6-磷酸果糖-2-激酶/果糖-2,6-二磷酸酶 3
1.755	BB2; CD54; P3.58	ICAM1	细胞间粘合分子 1 (CD54), 人类鼻病毒受体
1.742	BCL4; D19S37	BCL3	B-细胞 CLL/淋巴瘤 3
1.695	KRML; MGC43127	MAFB	v-maf 肌腱膜纤维肉瘤癌致癌基因类似物 B (鸟类的)
1.686	SRPSOX; CXCLG16; SR-PSOX	CXCL16	趋化因子 (C-X-C 基序) 配体 16
1.658	B3GN-T5; β3Gn-T5	B3GNT5	UDP-GlcNAc:βGal β-1,3-N-乙酰葡萄糖胺基转移酶 5
1.62	MLA1; ME491; LAMP-3; OMA81H; TSPAN30	CD63	CD63 分子
1.562	P21; CIP1; SDI1; WAF1; CAP20; CDKN1; MDA-6; p21CIP1	CDKN1A	细胞周期蛋白-依赖的激酶抑制剂 1A (p21, Cip1)
1.548	URAX1; TAIP-3; FAM130B; DKFZp566F164	AXUD1	AXIN1 上调的 1
1.542	NHE8; FLJ42500; KIAA0939; MGC138418; DKFZp686C03237	SLC9A8	溶质载体家族 9 (钠/氢交换剂), 成员 8
1.542	GS; GLNS; PIG43	GLUL	谷氨酸盐-氨连接酶 (谷氨酰胺合成酶)
1.504	CD87; UPAR; URKR	PLAUR	纤溶酶原激活物, 尿激酶受体

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相对的经校正的表达	普通名称	基因符号	描述
1.474	PBEF; NAMPT; MGC117256; DKFZP666B131; 1110035O14Rik	PBEF1	前 B 细胞群体增强因子 1
1.472	P47; FLJ27168	PLEK	普列克底物蛋白
1.45	GNA16	GNA15	鸟嘌呤核苷酸结合蛋白(G 蛋白), α 15 (Gq 类)
1.435	FTH; PLIF; FTHL6; PIG15; MGC104426	FTH1	铁蛋白、重多肽 1
1.42	MGC14376; MGC149751; DKFZp686O06159	MGC14376	待证实的蛋白质 MGC14376
1.395	NER; UNR; LXRB; LXR-b; NER-I; RIP15	NR1H2	核受体亚家族 1, 组 H, 成员 2
1.39	TTP; G0S24; GOS24; TIS11; NUP475; RNF162A	ZFP36	锌指蛋白 36, C3H 类型, 类似物(小鼠)
1.389	E4BP4; IL3BP1; NFIL3A; NF-IL3A	NFIL3	核因子, 白细胞介素 3 调节的
1.328	C8FW; GIG2; SKIP1	TRIB1	tribbles 类似物 1 (果蝇)
1.296	ARI; HARI; HHARI; UBCH7BP	ARIH1	ariadne 类似物, 泛素结合酶 E2 结合蛋白, 1 (果蝇)
1.272	FRA2; FLJ23306	FOSL2	FOS 样抗原 2
1.269	RIT; RIBB; ROC1; MGC125864; MGC125865	RIT1	无 CAAX 1 的 Ras 样
1.25	RBT1	SERTAD3	含有 SERTA 功能域的 3
1.227	MAPKAPK2	MAPKAPK2	有丝分裂活化的蛋白质激酶-活化的蛋白质激酶 2
1.217	PPG; PRG; PRG1; MGC9289; FLJ12930	PRG1	丝甘蛋白

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相对的经校正的表达	普通名称	基因符号	描述
1.181	SEI1; TRIP-Br1	SERTAD1	含有 SERTA 功能域的 1
1.172	CMT2; KIAA0110; MGC11282; RP1-261G23.6	MAD2L1 BP	MAD2L1 结合蛋白
1.169	UBP; SIH003; MGC129878; MGC129879	USP3	泛素特异性的肽酶 3

[0191] 表 7K M3.3PTBv. 对照，在活性的 TB 中过表现的基因。

[0192]

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相对的经校正的表达	普通名称	基因符号	描述
	P22_15_PTBvCSelect_09May08_PAL2Ttest_UP_M3.3		
3.651	MAYP; MGC34175	PSTPIP2	脯氨酸-丝氨酸-苏氨酸磷酸酶相互作用蛋白 2
3.2	Tiff66; MGC116930; MGC116931; MGC116932; MGC116933	VNN1	血管非炎性蛋白 1
2.604	Rsc6p; BAF60C; CRACD3; MGC111010	SMARCD3	SWI/SNF 相关的，基质相关的，染色质的肌动蛋白依赖的调节剂，亚家族 d，成员 3
2.157	FER1L1; LGMD2B; FLJ00175; FLJ90168	DYSF	dysferlin，肢带肌肉营养不良 2B (常染色体隐性)
2.091	ASRT5; IRAKM; IRAK-M	IRAK3	白细胞介素-1 受体-相关的激酶 3
2.082	p6; CAGC; CGRP; MRP6; CAAF1; ENRAGE	S100A12	S100 钙结合蛋白 A12
1.888	CGI-44	SQRDL	硫醌还原酶样 (酵母)

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相对的经校正的表达	普通名称	基因符号	描述
1.819	FAM31A; FLJ38464; KIAA1608; RP11-230L22.3	DENND1A	含有 DENN/MADD 功能域的 1A
1.736	APG3; APG3L; PC3-96; FLJ22125; MGC15201; DKFZp564M1178	ATG3	ATG3 自噬相关的 3 类似物 (酿酒酵母)
1.715	CAT1	CRAT	肉碱乙酰基转移酶
1.703	MGC2654; FLJ12433	MGC2654	染色体 16 开放阅读框 68
1.7	MD-2	LY96	淋巴细胞抗原 96
1.695	AD3; VRP; HBLP1	TBC1D8	TBC1 功能域家族, 成员 8 (具有 GRAM 功能域)
1.663	FLJ20424	C14orf94	染色体 14 开放阅读框 94
1.638	P28; GSTTLp28; DKFZp686H13163	GSTO1	谷胱甘肽 S-转移酶 ω1
1.635	ATRAP; MGC29646	AGTRAP	血管紧张素 II 受体-关联蛋白
1.572	FAT; GP4; GP3B; GPIV; CHDS7; PASIV; SCARB3	CD36	CD36 分子 (凝血酶致敏蛋白受体)
1.547	EI; LEI; PI2; MNEI; M/NEI; ELANH2	SERPINB1	丝氨酸蛋白酶抑制蛋白肽酶抑制剂, 进化枝 B (卵清蛋白), 成员 1
1.546	RAB32	RAB32	RAB32, 成员 RAS 致癌基因家族
1.541	CR3A; MO1A; CD11B; MAC-1; MAC1A; MGC117044	ITGAM	整合素, α M (补体成分 3 受体 3 亚单位)
1.481	ALFY; ZFYVE25; KIAA0993; MGC16461	WDFY3	含有 WD 重复和 FYVE 功能域的 3

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相对的经校正的表达	普通名称	基因符号	描述
1.467	ARHU; WRCH1; hG28K; CDC42L1; FLJ10616; DJ646B12.2; fJ646B12.2	RHOU	ras 类似物基因家族, 成员 U
1.459	SELR; SELX; MSRB1; HSPC270; MGC3344	SEPX1	硒蛋白 X, 1
1.432	LTA4H	LTA4H	白细胞三烯 A4 水解酶
1.409	VMP1; DKFZP566I133	TMEM49	跨膜蛋白 49
1.405	MGC33054	SNX10	分选连接蛋白 10
1.376	STX3A	STX3A	突触融合蛋白 3
1.369	TTG2; RBTN2; RHOM2; RBTNL1	LMO2	仅有 LIM 功能域 2 (菱形生物素样 1)
1.368	DBI; IBP; MBR; PBR; BZRP; PKBS; PTBR; mDRC; pk18	BZRP	转位分子蛋白质(18kDa)
1.361	CRE-BPA	CREB5	cAMP 响应元件结合蛋白 5
1.344	MAY1; MGC49908; nPKC-δ	PRKCD	蛋白质激酶 C, δ
1.341	AAA; AD1; PN2; ABPP; APPI; CVAP; ABETA; CTFγ	APP	淀粉样 β(A4)前体蛋白质(肽酶连接蛋白-II, 阿尔茨海默氏病)
1.333	CRFB4; CRF2-4; D21S58; D21S66; CDW210B; IL-10R2	IL10RB	白细胞介素 10 受体, β
1.31	DCIR; LLIR; DDB27; CLECSF6; HDCGC13P	CLEC4A	C-型凝集素功能域家族 4, 成员 A
1.304	HUFI-2; FLJ20248; FLJ22683;	LRRFIP2	富含亮氨酸重复的(在 FLII 中)相互作用蛋白 2

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相对的经校正的表达	普通名称	基因符号	描述
	DKFZp434H2035		
1.301	C32; CKLF1; CKLF2; CKLF3; CKLF4; UCK-1; HSPC224	CKLF	趋化因子样因子
1.289		ACSS2	
1.265	ESP-2; HED-2	ZYX	斑联蛋白
1.263	SH3BGR; MGC117402	SH3BGRL	结合 SH3 功能域的富含谷氨酸的蛋白质样
1.239	MTX; MTXN	MTX1	metaxin 1
1.237	ASC; TMS1; CARD5; MGC10332	PYCARD	含有 PYD 和 CARD 功能域的
1.233	a3; Stv1; Vph1; Atp6i; OC116; OPTB1; TIRC7; ATP6N1C; ATP6V0A3; OC-116kDa	TCIRG1	T 细胞，免疫调节剂 1，ATPase，H+ 转运，溶酶体 V0 亚单位 A3
1.223	JTK8; FLJ26625	LYN	v-yes-1 Yamaguchi 肉瘤病毒性相关的致癌基因类似物
1.209	GAIP; RGSGAIP	RGS19	G-蛋白质信号转导的调节剂 19
1.186	NEU; SIAL1	NEU1	唾液酸酶 1（溶酶体唾液酸酶）

[0196] 表 7L. M3.4PTBv. 对照，在活性的 TB 中低表现的基因。

[0197]

相对的经校正的表达	普通名称	基因符号	描述
	P22_15_PTBvCSelect_09May08_PAL2Ttest_DOWN_M3.4		
0.921	ZZZ4; FLJ10821;	ZZEF1	锌指，具有 EF 手功能域 1 的

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[0198]

相对的经校正的表达	普通名称	基因符号	描述
	FLJ45574; KIAA0399		ZZ 类型
0.905	TILZ4a; TILZ4b; TILZ4c; KIAA0669	TSC22D2	TSC22 功能域家族, 成员 2
0.891	XTP2; BAT2-iso	BAT2D1	含有 BAT2 功能域的 1
0.885	U2AF65	U2AF2	U2 小核 RNA 辅助因子 2
0.878	DKFZp781I24156	PCNP	PEST 含有蛋白水解信号的核蛋白
0.876	NY-CO-1; FLJ10051	SDCCAG1	血清学上定义的结肠癌抗原 1
0.868	GCP16; HSPC041; MGC4876; MGC21096; GOLGA3AP1	GOLGA7	高尔基自身抗原, golgin 亚家族 a, 7
0.866	CPR3; DJA2; DNAJ; DNJ3; RDJ2; HIRIP4; PRO3015	DNAJA2	DnaJ (Hsp40) 类似物, 亚家族 A, 成员 2
0.863	B2-1; SEC7; D17S811E; FLJ34050; FLJ41900; 胞黏蛋白-1	PSCD1	普列克底物蛋白类似, Sec7 和卷曲螺旋的功能域 1(胞黏蛋白 1)
0.855	SRrp86; SRrp508; MGC133045; DKFZp564B176	SFRS12	剪接因子, 富含精氨酸/丝氨酸的 12
0.84	G3BP2	G3BP2	GTPase 活化的蛋白(SH3 功能域)结合蛋白 2
0.831	p532; p619	HERC1	hect (类似 E6-AP (UBE3A) 羧基末端) 功能域和 RCC1 (CHC1)样功能域 (RLD) 1
0.826	DKFZP564O0523; HSPC304; DKFZp686D1651	DKFZP564O0523	待证实的蛋白质 DKFZp564O0523
0.823	TSPYL	TSPYL1	TSPY 样 1

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相对的经校正的表达	普通名称	基因符号	描述
0.82	KIP1; MEN4; CDKN4; MEN1B; P27KIP1	CDKN1B	细胞周期蛋白-依赖的激酶抑制剂 1B (p27, Kip1)
0.82	SA2; SA-2; FLJ25871; bA517O1.1; DKFZp686P168; DKFZp781H1753	STAG2	基质抗原 2
0.815	HR21; MCD1; NXP1; SCC1; hHR21; HRAD21; FLJ25655; FLJ40596; KIAA0078	RAD21	RAD21 类似物 (裂殖酵母)
0.808	GCC185; KIAA0336	GCC2	含有 GRIP 和卷曲螺旋功能域的 2
0.806	PIR1	DUSP11	双特异性磷酸酶 11 (RNA/RNP 复合物 1-相互作用)
0.804	AS3; CG008; PDS5B; FLJ23236; KIAA0979; RP1-267P19.1	APRIN	雄性激素诱导的增殖抑制剂
0.803		LOC58486	
0.798		SLTM	
0.795	AS; ANCR; E6-AP; HPVE6A; EPVE6AP; FLJ26981	UBE3A	泛素蛋白质连接酶 E3A (人类乳头瘤状病毒 E6 关联蛋白, Angelman 综合征)
0.793	DKFZp686C1054	THUMPD1	含有 THUMP 功能域的 1
0.791	SIR2L1	SIRT1	sirtuin (沉默交配型信息调控 2 类似物) 1 (酿酒酵母)
0.79	FLJ40359	TPP2	三肽基肽酶 II

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相对的经校正的表达	普通名称	基因符号	描述
0.789	DKFZP564D172	C5orf21	染色体 5 开放阅读框 21
0.788	PALBH; 钙蛋白酶 7; FLJ36423	CAPN7	钙蛋白酶 7
0.775	KIAA1116	RBM16	RNA 结合基序蛋白质 16
0.771	FLJ42355; KIAA0276	DCUN1D4	DCN1, 在 cullin neddylation 1 中缺陷的, 含有功能域的 4 (酿酒酵母)
0.768	Rhe; FLJ33619; DKFZp586K0717	FIP1L1	FIP1 样 1 (酿酒酵母)
0.766	RCP9; RCP; CRCP; CGRP-RCP; MGC111194	RCP9	降血钙素基因相关的肽-受体组分蛋白
0.764	DIF3; LZK1; DIF-3; LCRG1; ZFP403; FLJ21230; FLJ22561; FLJ42090	ZNF403	锌指蛋白 403
0.76	AD013; CReMM; KISH2; PRIC320	CHD9	染色质功能域螺旋酶 DNA 结合蛋白 9
0.757	VACM1; VACM-1	CUL5	cullin 5
0.755	MGC13407	NUP54	核孔蛋白 54kDa
0.751	ENTH; EPN4; EPNR; CLINT; EPSINR; KIAA0171	ENTH	网格蛋白相互作用物 1
0.743	SEC24B	SEC24B	SEC24 相关的基因家族, 成员 B (酿酒酵母); 同义词: SEC24, MGC48822; 同工型 a 由转录变体 1 编码; 分泌蛋白 24; Sec24 相关的蛋白质 B; 蛋白质转运蛋白质 Sec24B; 智人 SEC24 相关的基因家族, 成员 B (酿酒酵母) (SEC24B), 转录变体 1, mRNA。

[0201]

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相对的经校正的表达	普通名称	基因符号	描述
0.742	HAKAI; RNF188; FLJ23109; MGC163401; MGC163403	CBLL1	Cas-Br-M (鼠) 亲嗜性逆转录病毒转化序列样 1
0.738	XE7; 721P; XE7Y; CCDC133; CXYorf3; DXYS155E; MGC39904; MGC125365; MGC125366	DXYS155E	剪接因子, 富含精氨酸/丝氨酸的 17A
0.737	NGB; CRFG; FLJ10686; FLJ10690; FLJ39774	GTPBP4	GTP 结合蛋白 4
0.734	VELI3; LIN-7C; MALS-3; LIN-7-C; FLJ11215	LIN7C	lin-7 类似物 C (线虫)
0.732	JTK5; RYK1; JTK5A; D3S3195	RYK	RYK 受体样酪氨酸激酶
0.731	K10; KPP; CK10	KRT10	角蛋白 10 (表皮角化过多症; 掌跖角化病)
0.728	CYP-M; MGC22229	CYP20A1	细胞色素 P450,家族 20, 亚家族 A, 多肽 1
0.725	CHP1	CHORDC1	含有富含胱氨酸和组氨酸功能域 (CHORD)的 1
0.724	NET1A; ARHGEF8	NET1	神经上皮细胞转化基因 1
0.723	ZF5; ZBTB14; ZNF478; MGC126126	ZFP161	锌指蛋白 161 类似物 (小鼠)
0.718	JAK1A; JAK1B	JAK1	Janus 激酶 1 (蛋白质酪氨酸激酶)
0.717	p5; p6; RRP45; PMSCL1; Rrp45p;	EXOSC9	外来体元件 9

[0202]

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相对的经校正的表达	普通名称	基因符号	描述
	PM/Scl-75		
0.716	GR; GCR; GRL; GCCR	NR3C1	核受体亚家族 3, 组 C, 成员 1 (糖皮质激素受体)
0.713	L9mt	MRPL9	线粒体的核糖体蛋白质 L9
0.705	GRB1; p85-A	PIK3R1	磷酸肌醇-3-激酶, 调控亚单位 1 (p85 α)
0.7	MST4; MASK	MASK	丝氨酸/苏氨酸蛋白质激酶 MST4
0.7	UPF3; HUPF3A; RENT3A	UPF3A	UPF3 无意义转录物调节剂的类似物 A (酵母)
0.698	p17; YBL1; CHRAC17; CHARAC17	POLE3	聚合酶(DNA 定向的), epsilon 3 (p17 亚单位)
0.694	PCGF4; RNF51; MGC12685	PCGF4	BMI1 polycomb 无名指致癌基因
0.692	MIF2; CENPC; hcp-4; CENP-C	CENPC1	着丝点蛋白质 C 1
0.686	YAF9; GAS41; NUBI-1; 4930573H17Rik; B230215M10Rik	YEATS4	含有 YEATS 功能域的 4
0.679	R3HDM; FLJ23334; KIAA0029	R3HDM1	含有 R3H 功能域的 1
0.676	FBX21; FLJ90233; KIAA0875; MGC26682; DKFZp434G058	FBXO21	F-盒蛋白质 21
0.665	GRIPE; TULIP1; KIAA0884; DKFZp566D133; DKFZp667F074	GARNL1	GTPase 活化 Rap/RanGAP 功能域样 1
0.663	BRL; BRPF1; BRPF2;	BRD1	含有布罗莫结构域的 1

[0203]

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相对的经校正的表达	普通名称	基因符号	描述
	DKFZp686F0325		
0.651	TIFIA; MGC104238; DKFZp566E104	RRN3	RRN3 RNA 聚合酶 I 转录因子类似物 (酿酒酵母)
0.65	DKFZP586L0724	NOL11	核仁蛋白质 11
0.645	FLJ20628; DKFZp564I2178	FLJ20628	待证实的蛋白质 FLJ20628
0.642	FLJ21657; MGC90226; MGC149524	FLJ21657	染色体 5 开放阅读框 28
0.638	NS3TP1; FLJ20752; NBLA00058	ASNSD1	含有天冬酰胺合成酶功能域的 1
0.636	MEX3C; BM-013; MEX-3C; RNF194; FLJ38871	RKHD2	含有无名指和 KH 功能域的 2
0.628	E6BP; ERC55; ERC-55	RCN2	网钙结合蛋白 2, EF 手钙结合功能域
0.613	PHLL1	CRY1	隐花色素 1 (光裂合酶样)
0.612	cdc14; hCDC14; Cdc14A1; Cdc14A2	CDC14A	CDC14 细胞分裂周期 14 类似物 A (酿酒酵母)
0.576	LCA; LY5; B220; CD45; T200; GP180	PTPRC	蛋白质酪氨酸磷酸酶, 受体类型, C
0.521	PBF; PRF1; HDBP2; PRF-1; Si-1-8-14; DKFZp434K1210	ZNF395	锌指蛋白 395

[0204] 表 7M M3.6PTBv. 对照，在活性的 TB 中低表现的基因。

[0205]

相对的经校正的表达	普通名称	基因符号	描述
	P22_15_PTBvCSelect_09May08_PAL2Ttest		

[0206]

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相对的经校正的表达	普通名称	基因符号	描述
	_DOWN_M3.6		
0.898	ABHS; ORF20; TTDN1	C7orf11	染色体 7 开放阅读框 11
0.852	BTF2; TFIIH	GTF2H1	一般的转录因子 IIH, 多肽 1, 62kDa
0.845	MGC51029	FUNDC1	含有 FUN14 功能域的 1
0.844	SCOCO; HRIHFB2072	SCOC	短卷曲螺旋蛋白
0.839	IF-3mt; IF3(mt)	MTIF3	线粒体的翻译起始因子 3
0.816	DAB1; MPRP-1; YKR087C; ZMPOMA1; FLJ33782; 2010001O09Rik	OMA1	OMA1 类似物,锌金属肽酶(酿酒酵母)
0.815		LOC644560	
0.795	JNKK; MEK4; MKK4; SEK1; JNKK1; SERK1; MAPKK4; PRKMK4	MAP2K4	有丝分裂活化的蛋白质激酶激酶 4
0.775	REPA2; RPA32	RPA2	复制蛋白质 A2, 32kDa
0.765	AMMERC1	AMMECR1	Alport 综合征, 智力迟钝, 中脸发育不全和椭圆红细胞增多症染色体区域, 基因 1
0.741	CBX; M31; MOD1; HP1-BETA; HP1Hs-β	CBX1	染色盒类似物 1 (HP1 β 类似物 果蝇)
0.739	DLTA; PDCE2; PDC-E2	DLAT	二氢硫辛酰胺 S-乙酰基转移酶 (丙酮酸酯脱氢酶复合物的 E2 组分)
0.732	p38; AHA1; C14orf3	AHSA1	AHA1, 热激蛋白活化剂 90kDa 蛋白质 ATPase 类似物 1 (酵母)

[0207]

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相对的经校正的表达	普通名称	基因符号	描述
0.731	VEZATIN; DKFZp761C241	VEZT	vezatin, 粘着连接跨膜蛋白
0.728	HDPY-30	LOC84661	dpy-30 样蛋白质
0.727	DERP6; MST071; HSPC002; MSTP071	C17orf81	染色体 17 开放阅读框 81
0.723	EFG; GFM; EFG1; EFGM; EGF1; hEFG1; COXPD1; FLJ12662; FLJ13632; FLJ20773	GFM1	G 延长因子, 线粒体的 1
0.721	MGC3232; hAtNOS1; mAtNOS1	C4orf14	染色体 4 开放阅读框 14
0.72	P15RS; FLJ10656; MGC19513	P15RS	待证实的蛋白质 FLJ10656
0.719	MGC9912	C14orf126	染色体 14 开放阅读框 126
0.704	CCR4; KIAA1194	CNOT6	CCR4-NOT 转录复合体, 亚单位 6
0.7	PRED31; HSPC230; FLJ34245; RP11-59I9.1	C6orf203	染色体 6 开放阅读框 203
0.696	76P; GCP4	76P	γ 微管蛋白环状复合物蛋白质(76p 基因)
0.694	FLJ10422	ELP3	延长蛋白质 3 类似物 (酿酒酵母)
0.677	MGC13379	MGC13379	HSPC244
0.677	CCTE; KIAA0098; CCT-epsilon; TCP-1-epsilon	CCT5	含有 TCP1, 亚单位 5 (epsilon) 的伴侣蛋白
0.675		MTMR12	

[0208]

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相对的经校正的表达	普通名称	基因符号	描述
0.671	ABRA1; FLJ11520; FLJ12642; FLJ13614	FLJ13614	含有卷曲螺旋功能域的 98
0.671	CDG1; CDGS; CDG1a	PMM2	磷酸甘露糖变位酶 2
0.646	TPA1; FLJ10826; KIAA1612	OGFOD1	含有 2-氧代戊二酸盐和铁依赖的加氧酶功能域的 1
0.641	HV1; MGC15619	MGC15619	氢电压门通道 1
0.639	JJJ3; ZCSL3	ZCSL3	DPH4, JJJ3 类似物 (酿酒酵母)
0.631	GI008; RPMS13; MRP-S13; MRP-S26; NY-BR-87; C20orf193; dJ534B8.3	MRPS26	线粒体的核糖体蛋白质 S26
0.63	RPMS6; MRP-S6; C21orf101	MRPS6	线粒体的核糖体蛋白质 S6
0.622	CGI-55; CHD3IP; HABP4L; PAIRBP1; FLJ90489; PAI-RBP1; DKFZp564M2423	SERBP1	SERPINE1 mRNA 结合蛋白 1
0.621	MRP-S14; HSMRPS14; DJ262D12.2	MRPS14	线粒体的核糖体蛋白质 S14
0.542	LOC153364; MGC46734; DKFZp686P15118	LOC153364	类似于金属-β-内酰胺酶超家族蛋白

[0209] 表 7N M3.7PTBv. 对照，在活性的 TB 中低表现的基因。

[0210]

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相对的经校正的表达	普通名称	基因符号	描述
	P22_15_PTBvCSelect		

[0211]

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相对的经校正的表达	普通名称	基因符号	描述
	_09May08_PAL2Ttest_DOWN_M3.7		
0.914	RED; CSA2; MGC59741; IK 蛋白	IK	IK 细胞因子, HLA II 的下调剂
0.875	IBP	DEF6	在 FDCP 6 类似物 (小鼠)中有差别地表达
0.861	NAT3; dJ1002M8.1	NAT5	N-乙酰基转移酶 5
0.857	OFOXD; OFOXD1; FLJ20308	ALKBH5	alkB, 烷化修复类似物 5 (大肠杆菌)
0.848	H-IDHB; MGC903; FLJ11043	IDH3B	异柠檬酸脱氢酶 3 (NAD+) β
0.846	PGR1; PAM14	MRFAP1	Mof4 家族相关蛋白质 1
0.845	B17.2; DAP13	NDUFA12	NADH 脱氢酶 (泛醌) 1 α 次 subcomplex, 12
0.836	MGC11134	TRPT1	tRNA 磷酸转移酶 1
0.832	H-l(3)mbt-l	L3MBTL2	l(3)mbt 样 2 (果蝇)
0.831	HSCARG; FLJ25918	HSCARG	含有 NmrA 样家族功能域的 1
0.817	ABC27; ABC50	ABCF1	ATP- 结合盒, 亚家族 F (GCN20), 成员 1
0.816	LOC124512	LOC124512	待证实的蛋白质 LOC124512
0.815	HSPC203	C14orf112	染色体 14 开放阅读框 112
0.814	EXOSC1	EXOSC1	外切酶体元件 1; 同义词: p13, CSL4, SKI4, Csl4p, Ski4p, hCsl4p, CGI-108, RP11-452K12.9; 酵母外切酶体核心蛋白质 CSL4 类似物; 3'-5' 外切核糖核酸酶 CSL4 类似物; CSL4 外切酶体核心蛋白质类似物; 智人外切酶体

[0212]

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相对的经校正的表达	普通名称	基因符号	描述
			元件 1 (EXOSC1), mRNA。
0.81	p14; DOC-1R; FLJ10636	CDK2AP2	CDK2-相关蛋白质 2
0.81	MGC14833; bA6B20.2	C6orf125	染色体 6 开放阅读框 125
0.809	SRP68	SRP68	信号识别颗粒 68kDa
0.805	MGC3320; FLJ14936; RP5-965L7.1	PRPF38A	含有 PRP38 前 mRNA 处理因子 38 (酵母)的功能域的 A
0.805	DBP-RB; UKVH5d	DDX1	DEAD (Asp-Glu-Ala-Asp) 盒多肽 1
0.804	ACRP; FSA-1; MGC20134	SPAG7	精子相关抗原 7
0.802	MDHA; MOR2; MDH-s; MGC:1375	MDH1	苹果酸酯脱氢酶 1, NAD (可溶的)
0.801	MDS016; RPMS21; MRP-S21	MRPS21	线粒体的核糖体蛋白质 S21
0.8	AIBP; MGC119143; MGC119144; MGC119145	APOA1BP	载脂蛋白 A-I 结合蛋白
0.8	ERV29; FLJ22993; MGC102753	SURF4	surfeit 4
0.797	MGC874	CXorf26	染色体 X 开放阅读框 26
0.795	FLJ22789	C12orf26	染色体 12 开放阅读框 26
0.795	RC68; INT11; RC-68; INTS11; CPSF73L; FLJ13294; FLJ20542	CPSF3L	切割和聚腺苷酸特异性因子 3 样
0.793	HSPC196	HSPC196	跨膜蛋白 138
0.79	DS-1	ICT1	不成熟的结肠癌转录物 1
0.789	SIAHBP1; FIR; PUF60; RoBPI; FLJ31379	SIAHBP1	融合-结合蛋白-相互作用抑制物

[0213]

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相对的经校正的表达	普通名称	基因符号	描述
0.788	bMRP36a; MGC17989; MGC48892	MRPL43	线粒体的核糖体蛋白质 L43
0.788	HIT-17	HINT2	组氨酸三聚体核苷酸结合蛋白 2
0.785	MGC2714; FLJ32431	DCUN1D5	DCN1, 在 cullin neddylation 中缺陷的 1, 含有功能域的 5 (酿酒酵母)
0.784	WDC146; FLJ11294	WDR33	WD 重复功能域 33
0.775	N27C7-4; MGC70831	C22orf16	染色体 22 开放阅读框 16
0.774		LOC653709	
0.772	CGI-138; HSPC329; MRP-S23	MRPS23	线粒体的核糖体蛋白质 S23
0.769	P54; NMT55; NRB54; P54NRB	NONO	含有非-POU 功能域的, 八聚体结合的
0.764	NSE2; MMS21; C8orf36; FLJ32440	C8orf36	非-SMC 元件 2, MMS21 类似物 (酿酒酵母)
0.764	C8orf40	C8orf40	染色体 8 开放阅读框 40
0.763	FLJ31795	CCDC43	含有卷曲螺旋功能域的 43
0.755	NSE1	NSMCE1	非-SMC 元件 1 类似物 (酿酒酵母)
0.753	MY105; THY28; MDS012; HSPC144; THY28KD; MGC12187	THYN1	胸腺细胞核蛋白质 1
0.752	YSA1H; hYSAH1	NUDT5	nudix (核苷二磷酸酯连接的部分 X)-类基序 5
0.751	TOK-1	BCCIP	BRCA2 和 CDKN1A 相互作用蛋白
0.747	VARSL; VARS2L;	VARSL	缬氨酰-tRNA 合成酶 2, 线粒

[0214]

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相对的经校正的表达	普通名称	基因符号	描述
	MGC138259; MGC142165		体的 (推定的)
0.732	FLJ13657; RP11-337A23.1	C9orf82	染色体 9 开放阅读框 82
0.728	GLOD2	MCEE	甲基丙二酰 CoA 差向异构酶
0.728	C40	C2orf29	染色体 2 开放阅读框 29
0.726	MGC12966	MGC12966	待证实的蛋白质 LOC84792; 智人待证实的蛋白质 LOC84792 (MGC12966), mRNA。
0.722	FLJ14803	FLJ14803	待证实的蛋白质 FLJ14803
0.717	HSPC335; MRP-S24	MRPS24	线粒体的核糖体蛋白质 S24
0.716	RALBP1	REPS1	含有 RALBP1 关联的 Eps 功能域的 1
0.712	CAF1; hCAF-1	CNOT7	CCR4-NOT 转录复合物, 亚单位 7
0.711	A1U; UBIN; C1orf6	UBQLN4	泛醌蛋白 4
0.71	CGI-118; MGC13323	MRPL48	线粒体的核糖体蛋白质 L48
0.701	Gm83; HSPC064; MGC126859; MGC138247; DKFZP564O0463	WDSOF1	含有 WD 重复和 SOF1 功能域的
0.701	FMT1	MTFMT	线粒体的甲二硫酰基-tRNA 甲酸基转移酶
0.697	DKFZp686E10109	NUDCD2	含有 NudC 功能域的 2
0.697	MGC11321	MRPL45	线粒体的核糖体蛋白质 L45
0.691	SDOS; MGC11275	NUDT16L1	nudix (核苷二磷酸酯连接的部分 X)-类基序 16 样 1
0.683	FLJ20989	C8orf33	染色体 8 开放阅读框 33
0.681	AK6; FIX; AK3L1; AKL3L; AKL3L1	AK3	腺苷酸激酶 3

[0215]

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相对的经校正的表达	普通名称	基因符号	描述
0.671	RIP; HRIP; MGC4189	RIP	RPA 相互作用蛋白
0.666	PRP8; RP13; HPRP8; PRPC8	PRPF8	PRP8 前 mRNA 处理因子 8 类似物 (酿酒酵母)
0.664	PCMT; PPMT; PCCMT; HSTE14; MST098; MSTP098; MGC39955	ICMT	异戊烯半胱氨酸羧基甲基转移酶
0.66	YTM1; FLJ10881; FLJ12719; FLJ12720	WDR12	WD 重复功能域 12
0.646	GAB1; CDC91L1; MGC40420	CDC91L1	磷脂酰肌醇多糖锚定生物合成, U 类
0.613	MGC4248	C10orf58	染色体 10 开放阅读框 58
0.613	sen15	C1orf19	染色体 1 开放阅读框 19
0.599	MGC2404	ACBD6	含有酰基-辅酶 A 结合功能域的 6

[0216] 表 70 M3.8PTBv. 对照, 在活性的 TB 中低表现的基因。

[0217]

相对的经校正的表达	普通名称	基因符号	描述
	P22_15_PTBvCSelect_09May08_PAL2Ttest_DOWN_M3.8		
0.841	MAP; RUSC3; SGSM3; DKFZp761D051	RUTBC3	含有 RUN 和 TBC1 功能域的 3
0.84	FLJ13848	FLJ13848	N-乙酰基转移酶 11
0.827	HEL308; MGC20604	HEL308	DNA 螺旋酶 HEL308
0.826	dgkd-2; DGKδ; KIAA0145	DGKD	二乙酰基甘油激酶, δ 130kDa
0.814	DKFZp779L2418	SFRS14	剪接因子, 富含精氨酸/丝氨

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相对的经校正的表达	普通名称	基因符号	描述
			酸的 14
0.814	HMMH; MUTM; OGH1; HOGG1	OGG1	8-氧鸟嘌呤 DNA 糖基化酶
0.808	PRO9856; LAVS3040; DKFZp434D0711; DKFZp686L0539	BRD9	含有染色质域的 9
0.807	HCDI	C14orf124	染色体 14 开放阅读框 124
0.798	GTF2D; SCA17; TFIID; GTF2D1; MGC117320; MGC126054; MGC126055	TBP	TATA 盒结合蛋白
0.772	ZIS; ZIS1; ZIS2; ZNF265; FLJ41119; DKFZp686J1831; DKFZp686N09117	ZNF265	锌指，含有 RAN-结合功能域的 2
0.764		OGT	
0.762	MTMR8; C8orf9; LIP-STYX; MGC126672; DKFZp434K171	MTMR9	肌管蛋白相关蛋白 9
0.76	TDP-43	TARDBP	TAR DNA 结合蛋白
0.754	FPM315; ZKSCAN12	ZNF263	锌指蛋白 263
0.754	C42; CGI-05; HSPC167; C20orf34; CDK5RAP1.3; CDK5RAP1.4	CDK5RAP1	CDK5 调控亚单位相关蛋白 1
0.747	P50; P85; PAK3; PIXB; COOL1; P50BP; P85SPR; BETA-PIX;	ARHGEF7	Rho 鸟嘌呤核苷酸交换因子 (GEF) 7

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相对的经校正的表达	普通名称	基因符号	描述
	KIAA0142; KIAA0412; P85COOL1; Nbla10314; DKFZp761K1021		
0.745	NAC; CARD7; NALP1; SLEV1; DEFCAP; PP1044; VAMAS1; CLR17.1; KIAA0926; DEFCAP-L/S; DKFZp586O1822	NALP1	NLR 家族, 含有 pyrin 功能域的 1
0.744	KIAA0388	EZH1	zeste 类似物 1 (果蝇)的增强子
0.741	MGC19570; dJ34B21.3	C6orf130	染色体 6 开放阅读框 130
0.737	RP11-336K24.1	KIAA0907	KIAA0907
0.732	LAM; TSC; KIAA0243; MGC86987	TSC1	结节性硬化症 1
0.725	LRS; LEUS; LARS1; LEURS; PIG44; RNTLS; HSPC192; hr025Cl; FLJ10595; FLJ21788; KIAA1352	LARS	亮氨酰-tRNA 合成酶
0.724	HZF1	ZNF266	锌指蛋白 266
0.72	FAC1; FALZ; NURF301	FALZ	染色质域 PHD 指转录因子
0.72	FLJ12892; FLJ41065; DKFZp434L1050	CCDC14	含有卷曲螺旋功能域的 14
0.708	TIR8; MGC110992	SIGIRR	单个的免疫球蛋白和 toll-白细

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相对的经校正的表达	普通名称	基因符号	描述
			胞介素 1 受体 (TIR) 功能域
0.7	FLJ21007; RP11-459E2.1	TDRD3	含有 tudor 功能域的 3
0.691	CGI75; mtTFB; CGI-75	TFB1M	转录因子 B1, 线粒体的
0.689	FP977; FLJ12270; MGC11230	WDR59	WD 重复功能域 59
0.684	TS11	ASNS	天冬酰胺合成酶
0.677	MGC111199	NIT2	腈水解酶家族, 成员 2
0.675		ASB1	
0.663	MCAF2; FLJ12668	ATF7IP2	活化转录因子 7 相互作用蛋白 2
0.648	SIN; RPC5	POLR3E	聚合酶 (RNA) III (DNA 定向的) 多肽 E (80kD)
0.646	BMS1L; KIAA0187	BMS1L	BMS1 类似物, 核糖体组装蛋白质(酵母)
0.636	CBX7	CBX7	染色盒类似物 7
0.63	PAN2; hPAN2; FLJ39360; KIAA0710	USP52	泛素特异性的肽酶 52
0.623	MSK1; RLPK; MSPK1; MGC1911	RPS6KA5	核糖体蛋白质 S6 激酶, 90kDa, 多肽 5
0.612	SYB1; VAMP-1; DKFZp686H12131	VAMP1	囊泡关联的膜蛋白 1 (小突触小泡蛋白 1)
0.601	ALC1; CHDL; FLJ22530	CHD1L	染色质域螺旋酶 DNA 结合蛋白 1 样
0.587	KIAA0355	KIAA0355	KIAA0355
0.557	KIAA1615	ZNF529	锌指蛋白 529
0.554	MGC2146	IL11RA	白细胞介素 11 受体, α
0.552	RNF84; MGC:39780	TRAF5	TNF 受体关联因子 5
0.551	FLJ11795;	FLJ11795	锚蛋白重复功能域 55

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相对的经校正的表达	普通名称	基因符号	描述
	MGC126013; MGC126014		
0.548	DKFZp686O1788	MTX3	metaxin 3
0.544	DABP	DBP	白蛋白启动子的 D 位点(白蛋白 D-盒) 结合蛋白
0.541	FISH; SH3MD1	SH3PXD2A	SH3 和 PX 功能域 2A
0.524	CLAX; LLT1; OCIL	CLEC2D	C-型凝集素功能域家族 2, 成员 D
0.518	HPF1; FLJ11015; FLJ14876; FLJ90585; MGC33853	ZNF83	锌指蛋白 83
0.514	ZCW4; ZCWCC2; FLJ11565; dJ75H8.2	MORC4	MORC 家族 CW-型锌指 4
0.512	RTS; TYMSAS; RTS β; HSRTSBETA; RTS α	ENOSF1	烯醇化酶超家族成员 1
0.483	C7orf32; ATP6V0E2L	ATP6V0E2L	ATPase, H+运输 V0 亚单位 e2
0.458	PLC1; PLC-II; PLC148; PLCγ1	PLCG1	磷脂酶 C, γ 1
0.428	RLK; TKL; BTKL; PTK4; PSCTK5; MGC22473	TXK	TXK 酪氨酸激酶
0.367	T14; S152; Tp55; TNFRSF7; MGC20393	TNFRSF7	CD27 分子

[0222] 表 7P M3.9PTBv. 对照，在活性的 TB 中低表现的基因。

[0223]

相对的经校正的表达	普通名称	基因符号	描述

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[0224]

相对的经校正的表达	普通名称	基因符号	描述
	P22_15_PTBvCSelect_09May08_PAL2Ttest_DOWN_M3.9		
0.869	ABC43; PMP70; PXMP1	ABCD3	ATP-结合盒,亚家族 D (ALD), 成员 3
0.86	SPG8; MGC111053	KIAA0196	KIAA0196
0.859	PUMH; HSPUM; PUMH1; PUML1; KIAA0099	PUM1	pumilio 类似物 1 (果蝇)
0.856	ASF; SF2; SF2p33; SRp30a; MGC5228	SFRS1	剪接因子, 富含精氨酸/丝氨酸的 1 (剪接因子 2, 间隔的剪接因子)
0.848	DKFZp779N2044	KIAA0528	KIAA0528
0.843	ALG6	ALG6	天冬酰胺连接的糖基化 6 类似物 (酿酒酵母, α-1,3-葡萄糖基转移酶)
0.829	MGC111579; DKFZp781B11202	DARS	天冬氨酰基-tRNA 合成酶
0.829	ADDL	ADD3	内收蛋白 3 (γ)
0.829	KOX18; ZNF36; PHZ-37; ZNF139; MGC138429; 9130423L19Rik	ZKSCAN1	具有 KRAB 和 SCAN 功能域 1 的锌指
0.826	RPD3; YAF1	HDAC2	组蛋白脱乙酰基酶 2
0.825	FLJ21634; MGC71630	GALNT11	UDP-N- 乙酰基 -α-D- 半乳糖胺:多肽 N- 乙酰基半乳糖胺基转移酶 11 (GalNAc-T11)
0.816	POLZ; REV3	REV3L	REV3 样, DNA 聚合酶 ζ (酵母) 的催化亚单位
0.812	Ki; PA28G; REG-Γ;	PSME3	蛋白酶体 (前体, 巨蛋白因

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相对的经校正的表达	普通名称	基因符号	描述
	PA28-γ		子)活化剂亚单位 3 (PA28 γ; Ki)
0.811	BRM; SNF2; SWI2; hBRM; Sth1p; BAF190; SNF2L2; SNF2LA; hSNF2a; FLJ36757; MGC74511	SMARCA2	SWI/SNF 相关的，基质相关的，肌动蛋白依赖的染色质调控剂，亚家族 a，成员 2
0.807	ZNT5; ZTL1; ZNTL1; ZnT-5; MGC5499; FLJ12496; FLJ12756	SLC30A5	溶质载体家族 30 (锌载体)，成员 5
0.802	RAB7L; DKFZp686P1051	RAB7L1	RAB7，成员 RAS 致癌基因家族样 1
0.796	ASCIZ; KIAA0431; DKFZp779K1455	ASCIZ	ATM/ATR-底物 Chk2-相互作用 Zn2+-指蛋白
0.796	TAF2B; CIF150; TAFII150	TAF2	TAF2 RNA 聚合酶 II，TATA 盒结合蛋白(TBP)-关联因子，150kDa
0.786	N4WBP5; MGC10924	NDFIP1	Nedd4 家族相互作用蛋白 1
0.782	PAP41; MGC117304; MGC126719; MGC126721	PRPSAP2	磷酸核糖焦磷酸合成酶-关联蛋白 2
0.779	FLJ22584	TTC13	三十四肽重复功能域 13
0.775	CLCI; ICln; CLNS1B	CLNS1A	氯化物通道，核苷酸敏感的，1A
0.772	LRRC5; FLJ10470; FLJ20403	LRRC8D	含有富含亮氨酸的重复 8 的家族，成员 D
0.77	CCT6; Cctz; HTR3; TCPZ; TCP20; MoDP-2; TTCP20; CCT-ζ; MGC126214;	CCT6A	含有 TCP1 的伴侣蛋白，亚单位 6A (ζ 1)

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相对的经校正的表达	普通名称	基因符号	描述
	MGC126215; CCT-ζ-1; TCP-1-ζ		
0.765	TOK-1	BCCIP	BRCA2 和 CDKN1A 相互作用蛋白
0.764	G3BP; HDH-VIII; MGC111040	G3BP	GTPase 活化蛋白(SH3 功能域) 结合蛋白 1
0.763	FACT; CDC68; FACTP140; FLJ10857; FLJ14010; FLJ34357; SPT16/CDC68	SUPT16H	Ty 16 类似物的抑制物 (酿酒酵母)
0.757	FBP2; FLJ12799; FLJ38170	C14orf135	染色体 14 开放阅读框 135
0.753	GCP3; SPBC98; Spc98p	TUBGCP 3	微管蛋白，γ 复合物关联蛋白 3
0.752	FLJ13576; DKFZp564C012	FLJ13576	跨膜蛋白 168
0.751	SRP72	SRP72	信号识别颗粒 72kDa
0.75	CIA1; WDR39	WDR39	胞液的铁硫蛋白装配物 1 类似物 (酿酒酵母)
0.738	HPT; MRS2; MGC78523	MRS2L	MRS2 样，镁稳态因子(酿酒酵母)
0.729	CED-4; FLASH; RIP25; FLJ11208; KIAA1315	CASP8AP 2	CASP8 关联蛋白 2
0.728	PTPLB	PTPLB	蛋白质酪氨酸磷酸酶样 (脯氨酸替代催化的精氨酸)，成员 b
0.724	CHAC; FLJ42030; KIAA0986	VPS13A	液泡蛋白质分选 13 类似物 A (酿酒酵母)
0.724	REC14	WDR61	WD 重复功能域 61

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相对的经校正的表达	普通名称	基因符号	描述
0.719	EB9; PDAF; RCAS1	EBAG9	雌激素受体结合位点关联的，抗原，9
0.712	SNX4	SNX4	分选连接蛋白 4
0.704	TOPIIB; top2β	TOP2B	拓扑异构酶 (DNA) II β 180kDa
0.704	CGI-12; FLJ10939	MTERFD1	含有 MTERF 功能域的 1
0.703	CBC2; NIP1; CBP20; PIG55	NCBP2	核帽结合蛋白亚单位 2，20kDa
0.702	HAD; HHF4; HADH1; SCHAD; HADHSC; M/SCHAD; MGC8392	HADHSC	羟基乙酰基-辅酶 A 脱氢酶
0.701	p56; HSD8; FLJ11088; DKFZP779L1558; DKFZp779L1558	DKFZP779L1558	含有卷曲螺旋功能域的 91
0.701	CREB; MGC9284	CREB1	cAMP 应答元件结合蛋白 1
0.7	AIP5; Tiul1; hSDRP1; DKFZp434D2111	WWP1	含有 WW 功能域的 E3 泛素蛋白连接酶 1
0.681	TAT-SF1; dJ196E23.2	HTATSF1	HIV-1 Tat 特异性因子 1
0.674	LDLC	COG2	寡聚高尔基复合物 2 的组分
0.671	HC71; CGI-150; C17orf25	C17orf25	含有乙二醛酶功能域的 4
0.67	GABAT; NPD009; GABA-AT	ABAT	4-氨基丁酸转氨酶
0.668	AKAP18	AKAP7	A 激酶 (PRKA) 锚蛋白 7
0.661	LSFC; GP130; LRP130; CLONE-23970	LRPPRC	含有富含亮氨酸的 PPR-基序

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相对的经校正的表达	普通名称	基因符号	描述
0.644	SCC-112; PIG54; FLJ41012; KIAA0648; MGC131948; MGC161503; DKFZp686B19246	SCC-112	SCC-112 蛋白
0.643	GDE	AGL	淀粉-1, 6-葡萄糖苷酶, 4-α-葡萄糖基转移酶 (糖元去枝化酶, 糖原贮积病III型)
0.643	NIP3	BNIP3	BCL2/腺病毒 E1B 19kDa 相互作用蛋白 3
0.64	HSSB; RF-A; RP-A; REPA1; RPA70	RPA1	复制蛋白 A1, 70kDa
0.63	TAF2C; TAF4A; TAF2C1; FLJ41943; TAFII130; TAFII135	TAF4	TAF4 RNA 聚合酶 II, TATA 盒结合蛋白(TBP)关联因子, 135kDa
0.626	TMP21; S31I125; Tmp-21-I; S31III125; P24(DELTA)	TMED10	跨膜 emp24 样运输蛋白 10 (酵母)
0.617	FLJ20397; FLJ25564; FLJ31671; FLJ39381	FLJ20397	含有 HEAT 重复的 2
0.612	CHA; Figlb; E2BP-1; MGC46135	TCFL5	转录因子样 5 (碱性/螺旋-环-螺旋)
0.588	SRB; Cctd; MGC126164; MGC126165	CCT4	含有 TCP1 的伴侣蛋白, 亚单位 4 (δ)
0.582	Seh1; SEH1A; SEH1B; SEC13L	SEH1L	SEH1 样 (酿酒酵母)
0.527	HSU79274	C12orf24	染色体 12 开放阅读框 24

[0229] 表 8A M1.5LTBv. 对照,在潜伏性的 TB 中低表现的基因。

[0230]

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相对的经校正的表达	普通名称	基因符号	描述
	P22_15_LTBvCSelect_09May08_PAL2Ttest_DOWN_M1.5		
2.007	STF1; STFA	CSTA	半胱氨酸蛋白酶抑制剂 A (stefin A)
1.915	LSH; NRAMP; NRAMP1	SLC11A1	溶质载体家族 11 (质子偶联的二价金属离子转运剂), 成员 1
1.903	EZI; Zfp467	ZNF467	锌指蛋白 467
1.813	TIL4; CD282	TLR2	toll-类似受体 2
1.811	HSULF-2; FLJ90554; KIAA1247; MGC126411; DKFZp313E091	SULF2	硫酸酯酶 2
1.716	FLJ22662	FLJ22662	待证实的 蛋白质 FLJ22662
1.691	FDF03	PILRA	配对的免疫球蛋白样类型 2 受体 α
1.686	HET; ITM; BWR1A; IMPT1; TSSC5; ORCTL2; BWSCR1A; SLC22A1L; p45-BWR1A; DKFZp667A184	SLC22A18	溶质载体家族 22 (有机阳离子转运剂), 成员 18
1.682	ILT1; LIR7; CD85H; LIR-7	LILRA2	白细胞免疫球蛋白-样受体, 亚家族 A (具有 TM 功能域), 成员 2
1.657	C1QR1; C1qRP; CDw93; MXRA4; C1qR(P); dJ737E23.1	C1QR1	CD93 分子

[0231]

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相对的经校正的表达	普通名称	基因符号	描述
1.636	NCF; MGC3810; P40PHOX; SH3PXD4	NCF4	嗜中性粒细胞的胞液因子 4, 40kDa
1.623	NOXA2; p67phox; P67-PHOX	NCF2	嗜中性粒细胞的胞液因子 2 (65kDa, 慢性肉芽肿病, 常染色体 2)
1.542	FLJ10357; SOLO	FLJ10357	待证实的蛋白质 FLJ10357
1.525	JTK9	HCK	造血细胞激酶
1.521	FEM-2; POPX2; hFEM-2; CaMKPase; KIAA0015	PPM1F	蛋白质磷酸酶 1F (含有 PP2C 功能域)
1.498	CD32; FCG2; FcGR; CD32A; CDw32; FCGR2; IGFR2; FCGR2A1; MGC23887; MGC30032	FCGR2A	IgG 的 Fc 片段, 低亲和性 IIa, 受体 (CD32)
1.493	DHRS8; PAN1B; RETSDR2; 17-BETA-HSD11; 17-BETA-HSDXI	DHRS8	羟基固醇(17-β) 脱氢酶 11
1.482	FLJ11151; CSTP1	FLJ11151	待证实的蛋白质 FLJ11151
1.478	CD31; PECAM-1	PECAM1	血小板/内皮的细胞粘合分子 (CD31 抗原)
1.469	DORA	IGSF6	免疫球蛋白超家族, 成员 6
1.452	GP; G1RZFP; GOLIATH; MGC99542; MGC117241; MGC138647	RNF130	环指蛋白质 130
1.45	MLN70; S100C	S100A11	S100 钙结合蛋白 A11
1.449	MGC3886	CTSS	组织蛋白酶 S
1.425	APPH; APPL2;	APLP2	淀粉样 β (A4) 前体样蛋白质

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相对的经校正的表达	普通名称	基因符号	描述
	CDEBP		2
1.41	IMPD; RP10; IMPD1; LCA11; sWSS2608; DKFZp781N0678	IMPDH1	IMP (肌苷单磷酸酯) 脱氢酶 1
1.406	FCNM	FCN1	ficolin (含有胶原蛋白/纤维原蛋白功能域的) 1
1.376	MYD88	MYD88	髓样分化原初应答基因 (88)
1.371	B144; LST-1; D6S49E; MGC119006; MGC119007	LST1	白细胞特异性的转录物 1
1.348	OS9	OS9	在骨肉瘤中扩增
1.334	TEM7R; FLJ14623	PLXDC2	含有 plexin 功能域的 2
1.334	Rab22B	RAB31	RAB31, 成员 RAS 致癌基因家族
1.301	TS; TXS; CYP5; THAS; TXAS; CYP5A1	TBXAS1	凝血噁烷 A 合成酶 1 (血小板, 细胞色素 P450,家族 5, 亚家族 A)
1.292	HXK3; HKIII	HK3	己糖激酶 3 (白细胞)
1.292	RISC; HSCP1	SCPEP1	丝氨酸羧肽酶 1
1.283	IBA1; AIF-1; IRT-1	AIF1	异基因移植物炎症因子 1
1.283	CD14	CD14	CD14 分子
1.27	PI; A1A; AAT; PI1; A1AT; MGC9222; PRO2275; MGC23330	SERPINA1	丝氨酸蛋白酶抑制蛋白肽酶抑制剂, 进化枝 A (α-1 抗蛋白酶, 抗酪蛋白), 成员 1
1.261	LIR6; CD85I; LIR-6; MGC126563	LILRA1	白细胞免疫球蛋白-样受体, 亚家族 A (具有 TM 功能域), 成员 1
1.221	CAP102; FLJ36832	CTNNA1	联蛋白(钙黏着蛋白关联蛋白), α 1, 102kDa

[0233]

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相对的经校正的表达	普通名称	基因符号	描述
1.192	BCKDK	BCKDK	支链酮酸脱氢酶激酶
1.137	p75; TBPII; TNFBR; TNFR2; CD120b; TNFR80; TNF-R75; p75TNFR; TNF-R-II	TNFRSF1B	肿瘤坏死因子受体超家族，成员 1B

[0234] 表 8B M2. 1LTBv. 对照，在潜伏性的 TB 中过表现的基因。

[0235]

相对的经校正的表达	普通名称	基因符号	描述
	P22_15_LTBvCSelect_09May08_PAL2Ttest_UP_M2.01		
0.801	LIME; LP8067; FLJ20406; dJ583P15.4; RP4-583P15.5	LIME1	Lck 相互作用的跨膜接头 1
0.769	FLJ34563; MGC35163	SAMD3	含有 sterile α 基序功能域的 3
0.763	SISd; SCYA5; RANTES; TCP228; D17S136E; MGC17164	CCL5	趋化因子 (C-C 基序) 配体 5
0.758	ORP7; MGC71150	OSBPL7	氧类固醇结合蛋白样 7
0.757		LOC387882	
0.736	SLP2; SGA72M; CHR11SYT; KIAA1597; MGC102768	SYTL2	突触结合蛋白样 2
0.735	DORZ1; DKFZP564O243	ABHD14A	含有 ab 水解酶功能域的 14A

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0.727	MGC33870; MGC74858	NCALD	神经钙蛋白 δ
0.691	LPAP; CD45-AP; MGC138602; MGC138603	PTPRCAP	蛋白质酪氨酸磷酸酶, 受体类型, C-关联蛋白
0.686	T11; SRBC	CD2	CD2 分子
0.671	CD8; MAL; p32; Leu2	CD8A	CD8a 分子
0.656	HOP; OB1; LAGY; Toto; Cameo; NECC1; SMAP31; MGC20820	HOP	仅有同源域的蛋白
0.651	2F1; MAFA; MAFA-L; CLEC15A; MAFA-2F1; MGC13600	KLRG1	杀伤细胞凝集素-样受体亚家族 G, 成员 1
0.65		LOC197135	
0.643	GIG1	NKG7	天然杀伤细胞 7 组序列
0.638	TSAd; F2771	SH2D2A	SH2 功能域蛋白质 2A
0.634	FEOM; CFEOM; FEOM1; CFEOM1; FLJ20052; KIAA1708; DKFZp779C159	KIF21A	驱动蛋白家族成员 21A
0.627	KIAA0442; MGC13140	AUTS2	孤独症感受候选 2
0.583	BFPP; TM7LN4; TM7XN1; DKFZp781L1398	GPR56	G 蛋白-偶联受体 56
0.572	TARP; CD3G; TCRG; TCRGC1; TCRGC2	TARP	TCR γ 间隔阅读框蛋白
0.502	519; LAG2; NKG5; LAG-2; D2S69E; TLA519	GNLY	粒溶素
0.303	CCP-X; CGL-2;	GZMH	粒酶 H (组织蛋白酶 G 样 2,

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	CSP-C; CTLA1; CTSGL2		蛋白质 h-CCPX)

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[0238] 表 8C M2.6LTBv. 对照，在潜伏性的 TB 中低表现的基因。

[0239]

相对的经校正的表达	普通名称	基因符号	描述
	P22_15_LTBvCSelect_09May08_PAL2Ttest_DOWN_M2.06		
	模块 2.06，髓样，相对 LTB，成倍变化是有益健康的，即在 LTB 中下降		
2.409	HsT287	ZNF516	锌指蛋白 516
2.286	CRISP11; LCRISP2; MGC74865; DKFZP434B044	CRISPLD2	含有富含半胱氨酸的分泌蛋白 LCCL 功能域的 2
2.177	MAG1; GPAT3; AGPAT8; MGC11324	HMFN0839	肺癌转移关联蛋白
2.095	CDD	CDA	胞苷脱氨酶
2.094	CRBP4; CRBPIV; MGC70641	RBP7	视黄醇结合蛋白 7，细胞的
1.917	SSC1; HsT17287	AQP9	水通道蛋白 9
1.916	GMR; CD116; CSF2R; CDw116; CSF2RX; CSF2RY; GMCSFR; CSF2RAX; CSF2RAY; MGC3848; MGC4838; GM-CSF-R-α	CSF2RA	集落刺激因子 2 受体，α，低亲和性的 (粒细胞-巨噬细胞)
1.853	G0S8	RGS2	G-蛋白质信号 2 调节剂，24kDa

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1.734	HKII; HXK2; DKFZp686M1669	HK2	己糖激酶 2
1.734	BB1	LENG4	白细胞受体簇(LRC) 成员 4
1.701	UB1; CEP3; BORG2; FLJ46903	CDC42EP3	CDC42 效应蛋白(Rho GTPase 结合) 3
1.671	SPAL2; FLJ23126; FLJ23632; KIAA1389	SIPA1L2	信号诱导的增殖关联的 1 样 2
1.669	ST1; SYCL; MDA-9; TACIP18	SDCBP	多配体聚糖结合蛋白(内居蛋白)
1.669	CAN; CAIN; N214; D9S46E; MGC104525	NUP214	核孔蛋白 214kDa
1.651		SLC19A1	
1.65	LPB3; S1P3; EDG-3; S1PR3; FLJ37523; MGC71696	EDG3	内皮分化, 鞘脂 G-蛋白偶联受体, 3
1.642	FPR; FMLP	FPR1	甲酸基肽受体 1
1.61	GPCR1; GPR86; GPR94; P2Y13; SP174; FKSG77	P2RY13	嘌呤能受体 P2Y, G-蛋白偶联的, 13
1.606	WDR80; FLJ00012	ATG16L2	ATG16 自噬相关的 16 样 2 (酿酒酵母)
1.601	LENG5; SEN34; SEN34L	TSEN34	tRNA 剪接核酸内切酶 34 类似物 (酿酒酵母)
1.575	FPF; p55; p60; TBP1; TNF-R; TNFAR; TNFR1; p55-R; CD120a; TNFR55; TNFR60; TNF-R-I; TNF-R55; MGC19588	TNFRSF1A	肿瘤坏死因子受体超家族, 成员 1A
1.572	PELI2	PELI2	pellino 类似物 2 (果蝇)
1.562	FLJ13052; FLJ37724; dJ283E3.1; RP1-283E3.6	NADK	NAD 激酶

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1.558	5-LO; 5LPG; LOG5; MGC163204	ALOX5	花生四烯酸酯 5-脂肪氧合酶
1.534	TMPIT	TMPIT	由肿瘤坏死因子 α 诱导的跨膜蛋白
1.517	FLJ31978	GLT1D1	含有糖基转移酶 1 功能域的 1
1.517	PFKFB4	PFKFB4	6-磷酸果糖-2-激酶/果糖-2,6-二磷酸酶 4
1.516	FLJ22470; KIAA1993; MGC24652; RP11-106H5.1	ZBTB34	含有锌指和 BTB 功能域的 34
1.482	P39; VATX; VMA6; ATP6D; ATP6DV; VPATPD	ATP6V0D1	ATPase, H+ 运输, 溶酶体 38kDa, V0 亚单位 d1
1.473	PRAM-1; MGC39864	PRAM1	PML-RARA 调控的接头分子 1
1.471	BIT; MFR; P84; SIRP; MYD-1; SHPS1; CD172A; PTPNS1; SHPS-1; SIRPα; SIRPα2; SIRP-ALPHA-1	PTPNS1	信号-调控蛋白质 α
1.463	M130; MM130	CD163	CD163 分子
1.434	AF-1; IFGR2; IFNGT1	IFNGR2	干扰素 γ 受体 2 (干扰素 γ 转导器 1)
1.405	RALB	RALB	v-ral 类人猿白血病病毒性的致癌基因类似物 B (ras 相关的; GTP 结合蛋白)
1.405	SLCO3A1	SLCO3A1	溶质载体有机阴离子转运子家族, 成员 3A1; 同义词: OATP-D, OATP3A1, FLJ40478, SLC21A11; 溶质载体家族 21 (有机阴离子转运物), 成员 11; 智人溶质载体有机阴离子转运子家族, 成员

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			3A1 (SLCO3A1), mRNA。
1.397	PTPE; HPTPE; DKFZp313F1310; R-PTP-EPSILON	PTPRE	蛋白质酪氨酸磷酸酶，受体型，E
1.397	RCC4; FLJ14784	DIRC2	在肾癌中扰乱的 2
1.396	DAP12; KARAP; PLOSL	TYROBP	TYRO 蛋白质酪氨酸激酶 结合蛋白
1.371	B144; LST-1; D6S49E; MGC119006; MGC119007	LST1	白细胞特异性的转录物 1
1.359	BFD; PFC; PFD; PROPERDIN	PFC	补体因子备解素
1.31	CAG4A; ERDA5; PRAT4A	TNRC5	含有三核苷酸重复的 5
1.307	CD18; TNFCR; D12S370; TNFR-RP; TNFRSF3; TNFR2-RP; LT-BETA-R; TNF-R-III	LTBR	淋巴毒素 β 受体 (TNFR 超家族，成员 3)
1.305	CEB	VAMP3	囊泡关联的膜蛋白 3 (小细胞小泡蛋白)
1.304	CSC-21K	TIMP2	TIMP 金属肽酶抑制剂 2
1.301	BPOZ; EF1ABP; PP2259; MGC20585	ABTB1	含有锚蛋白重复和 BTB (POZ)功能域的 1
1.294	C6orf209; FLJ11240; bA810I22.1; RP11-810I22.1	LMBRD1	含有 LMBR1 功能域的 1
1.266	PBF; C21orf1; C21orf3	PTTG1IP	垂体瘤转化 1 相互作用蛋白
1.235	ZFYVE10; FLJ32333; KIAA0371; FYVE-DSP1	MTMR3	肌管蛋白相关蛋白 3
1.216	CFP1; CBCP1;	C10orf9	细胞周期蛋白 Y

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	C10orf9		
1.2	SPT4H; SUPT4H	SUPT4H1	Ty 4 类似物 1 的抑制物 (酿酒酵母)

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[0244] 表 8D M2.10LTBv. 对照，在潜伏性的 TB 中低表现的基因。

[0245]

相对的经校正的表达	普通名称	基因符号	描述
	P22_15_LTBvCSelect_09May08_PAL2Ttest_DOWN_M2.10		
	未定义的模块 M2.10，相比 LTB，成倍变化是有益健康的，即在 LTB 中下降		
1.608	JAML; AMICA; Gm638; CREA7-1; CREA7-4; FLJ37080; MGC118814; MGC118815	AMICA1	粘合分子，与 CXADR 抗原 1 相互作用
1.537	MPEG1; MGC132657; MGC138435	MPEG1	巨噬细胞表达的基因 1
1.514	L13; MGC13061	RNF135	环指蛋白质 135
1.507	PAKα; MGC130000; MGC130001	PAK1	p21/Cdc42/Rac1-活化的激酶 1 (STE20 类似物，酵母)
1.471	T49; pT49	FGL2	纤维蛋白原样 2
1.405	KIAA0513	KIAA0513	KIAA0513
1.396	NCKX4; SLC24A2; FLJ38852	SLC24A4	溶质载体家族 24 (钠/钾/钙交换剂)，成员 4
1.358	FLJ34389	MLKL	混合谱系的激酶功能域样

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相对的经校正的表达	普通名称	基因符号	描述
1.348	ETO2; MTG16; MTGR2; ZMYND4	CBFA2T3	核-结合因子, runt 功能域, α 亚单位 2; 易位到, 3
1.331	IRC1; IRC2; IRp60; IGSF12; CMRF35H; CMRF-35H; CMRF35H9; CMRF-35-H9	CD300A	CD300a 分子
1.3	GLIPR; RTVP1; CRISP7	GLIPR1	GLI 发病机制相关的 1 (神经胶质瘤)
1.229	ENC-1AS	HEXB	氨基己糖苷酶 B (β 多肽)
1.222	TIRP; TRAM; TIRAP3; TICAM-2; MGC129876; MGC129877	TICAM2	toll-类似受体接头分子 2
1.175	FLJ31265	NUDT16	nudix (核苷二磷酸酯连接的部分 X)-型基序 16
1.17	FKBP133; KIAA0674	KIAA0674	FK506 结合蛋白 15, 133kDa

[0247] 表 8B M3.2LTBv. 对照，在潜伏性的 TB 中低表现的基因。

[0248]

相对的经校正的表达	普通名称	基因符号	描述
	P22_15_LTBvCSelect_09May08_PAL2Ttest_DOWN_M3.2		
	相比 LTB，炎症 3.2 倍变化是有益健康的，也即在 LTB 中下		

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[0249]

相对的经校正的表达	普通名称	基因符号	描述
	降		
4.289	K60; NAF; GCP1; LECT; LUCT; NAP1; 3-10C; CXCL8; GCP-1; LYNAP; MDNCF; MONAP; NAP-1; SCYB8; TSG-1; AMCF-I; b-ENAP	IL8	白细胞介素 8
2.068	CD87; UPAR; URKR	PLAUR	纤溶酶原激活物，尿激酶受体
2.009	PBEF; NAMPT; MGC117256; DKFZP666B131; 1110035O14Rik	PBEF1	前体 B-细胞群体增强因子 1
1.9		IER3	
1.87	TREM-1	TREM1	在髓细胞中表达的触发受体 1
1.79	E4BP4; IL3BP1; NFIL3A; NF-IL3A	NFIL3	核因子，白细胞介素 3 调节的
1.739	KIAA1145	TMCC3	跨膜和卷曲螺旋功能域家族 3
1.728	PINH; FLJ21759; FLJ23500; C20orf110; dJ1181N3.1; DKFZp434B2411; DKFZp434O0827	TP53INP2	肿瘤蛋白质 p53 可诱导的核蛋白 2
1.705	MAD; MAD1; MGC104659	MXD1	MAX 二聚蛋白 1
1.657	SGK1	SGK	血清/糖皮质激素调控的激酶
1.654	SLCO3A1	SLCO3A1	溶质载体有机阴离子转运子家族，成员 3A1；同义词：OATP-D, OATP3A1, FLJ40478, SLC21A11；溶质载体家族 21 (有机阴离子转运

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相对的经校正的表达	普通名称	基因符号	描述
			子), 成员 11; 智人溶质载体有机阴离子转运子家族, 成员 3A1 (SLCO3A1), mRNA。
1.637	C5orf6	FAM53C	具有序列相似性 53 的家族, 成员 C
1.632	PDLIM7	PDLIM7	PDZ 和 LIM 功能域 7 (未知的)
1.591	NIN1; NINJURIN	NINJ1	ninjurin 1
1.572	RIT; RIBB; ROC1; MGC125864; MGC125865	RIT1	无 CAAX 1 的 Ras 样
1.567	SB135	MYADM	髓关联的分化标记物
1.54	RCP; NOEL1A; FLJ22524; FLJ22622; MGC78448; rab11-FIP1; DKFZp686E2214	RAB11FIP1	RAB11 家族相互作用蛋白 1 (I 类)
1.526	DANGER; bA127L20; bA127L20.2; RP11-127L20.4	KIAA1754	KIAA1754
1.515		SPAG9	
1.499	HSS; JLP; HLC4; PHET; PIG6; FLJ13450; FLJ14006; FLJ26141; FLJ34602; KIAA0516; MGC14967; MGC74461; MGC117291	SPAG9	精子关联抗原 9
1.496	MGC20461	OSM	制瘤素 M
1.444	KIAA1673	CPEB4	细胞质的聚腺苷酸元件结合蛋白 4

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相对的经校正的表达	普通名称	基因符号	描述
1.433	IL-1; IL1F2; IL1-B	IL1B	白细胞介素 1, β
1.413	TRIP8; FLJ14374; KIAA1380; RP11-10C13.2; DKFZp761F0118	JMJD1C	含有 jumonji 功能域的 1C
1.41	FLJ11080; FLJ33961; DKFZP566A1524	FAM49A	具有序列相似性 49 的家族, 成员 A
1.4	EOPA; NUDEL; MITAP1; DKFZp451M0318	NDEL1	nudE 核分布基因 E 类似物 (构巢曲菌)样 1
1.384	NHE8; FLJ42500; KIAA0939; MGC138418; DKFZp686C03237	SLC9A8	溶质载体家族 9 (钠/氢交换剂, 成员 8
1.379	FLJ14744	PPP1R15B	蛋白质磷酸酶 1, 调控(抑制剂) 亚单位 15B
1.356	PPG; PRG; PRG1; MGC9289; FLJ12930	PRG1	丝甘蛋白
1.348	ATG8; GEC1; APG8L	GABARAPL1	GABA(A)受体关联蛋白样 1
1.332	TTP; G0S24; GOS24; TIS11; NUP475; RNF162A	ZFP36	锌指蛋白 36, C3H 型, 类似物 (小鼠)
1.329	PFK2; IPFK2	PFKFB3	6-磷酸果糖-2-激酶/果糖-2,6-二磷酸酶 3
1.31	DKFZp547M072	MIDN	midnolin
1.301	FLJ13448	COQ10B	辅酶 Q10 类似物 B (酿酒酵母)
1.285	C8FW; GIG2; SKIP1	TRIB1	tribbles 类似物 1 (果蝇)
1.284	FLJ13725; KIAA1930	FAM65A	具有序列类似性 65 的家族, 成员 A

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相对的经校正的表达	普通名称	基因符号	描述
1.272	FLJ46337; MGC117209; DKFZP434H132	C15orf39	染色体 15 开放阅读框 39
1.258	AII; AVP; FCU; MWS; FCAS; CIAS1; NALP3; C1orf7; CLR1.1; PYPAF1; AII/AVP; AGTAVPRL	CIAS1	NLR 家族，含有 pyrin 功能域的 3
1.252	BRF1; ERF1; cMG1; ERF-1; Berg36; TIS11B; RNF162B	ZFP36L1	锌指蛋白 36, C3H 型样 1
1.249	FRA2; FLJ23306	FOSL2	FOS 样抗原 2
1.235	GADD34	PPP1R15A	蛋白质磷酸酶 1，调控(抑制剂) 亚单位 15A
1.235	p33; p47; p33ING1; p24ING1c; p33ING1b; p47ING1a	ING1	生长家族的抑制剂，成员 1
1.231	P47; FLJ27168	PLEK	普列克底物蛋白
1.218	UBP; SIH003; MGC129878; MGC129879	USP3	泛素特异性的肽酶 3
1.208	Sei-2; TRIP-Br2; MGC126688; MGC126690	SERTAD2	含有 SERTA 功能域的 2
1.204	DCTN4	DCTN4	动力蛋白激活蛋白 4 (p62)
1.192	ROX; MAD6; MXD6	MNT	MAX 结合蛋白
1.165	RBT1	SERTAD3	含有 SERTA 功能域的 3
1.157	WIPI3; WIPI-3	WDR45L	WDR45 样
1.156	ERF; RF1; ERF1; TB3-1; D5S1995; SUP45L1; MGC111066	ETF1	真核翻译终止因子 1

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相对的经校正的表达	普通名称	基因符号	描述
1.156	KIAA0118	RAB21	RAB21, 成员 RAS 致癌基因家族
1.098	MAPKAPK2	MAPKAPK2	有丝分裂活化的蛋白质激酶-活化的蛋白质激酶 2

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[0254] 表 8F M3.3LTBv. 对照，在潜伏性的 TB 中低表现的基因

[0255]

相对的经校正的表达	普通名称	基因符号	描述
	P22_15_LTBvCSelect_09May08_PAL2Ttest_DOWN_M3.3		
	相比 LTB，炎症 3.2 倍变化是有益健康的，也即在 LTB 中下降		
2.716	QC; GCT	QPCT	谷氨酰肽环转移酶 (谷氨酰环化酶)
2.579	CRE-BPA	CREB5	cAMP 应答元件结合蛋白 5
2.468	APN; CD13; LAP1; PEPN; gp150	ANPEP	丙氨酰(膜) 氨基肽酶 (氨基肽酶 N, 氨基肽酶 M,微粒体氨基肽酶, CD13, p150)
2.426	PAD; PDI4; PDI5; PADI5	PADI4	肽基精氨酸脱亚胺酶，类型 IV
2.245	MRP; WLS; C1orf139; FLJ23091; MGC14878; MGC131760	GPR177	G 蛋白-偶联受体 177
2	HIS; HSTD; 组氨酸酶	HAL	组氨酸解氨酶
1.963	PYGL	PYGL	磷酸化酶，糖原；肝脏 (Hers 病, VI 型糖原贮存症)

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相对的经校正的表达	普通名称	基因符号	描述
1.948		EGFL5	
1.935	L-H2; ASGP-R; CLEC4H2; Hs.1259	ASGR2	无唾液酸糖蛋白受体 2
1.892	CD114; GCSFR	CSF3R	集落刺激因子 3 受体 (粒细胞)
1.882	LAMPB; CD107b; LAMP-2C	LAMP2	溶酶体关联的膜蛋白 2
1.813	ALFY; ZFYVE25; KIAA0993; MGC16461	WDFY3	含有 WD 重复和 FYVE 功能域的 3
1.8	STX3A	STX3A	突触融合蛋白 3
1.771	CR1	CR1	补体组分 (3b/4b) 受体 1 (Knops 血型); 同义词: KN, C3BR, CD35; 同工型 F 前体由转录变体 F 编码; C3-结合蛋白; CD35 抗原; 补体组分受体 1; C3b/C4b 受体; Knops 血型抗原; 智人补体组分 (3b/4b) 受体 1 (Knops 血型) (CR1), 转录变体 F, mRNA。
1.764	DCL-1; BIMLEC; CLEC13A; KIAA0022	CD302	CD302 分子
1.758	FER1L1; LGMD2B; FLJ00175; FLJ90168	DYSF	dysferlin, 肢带型肌营养不良症 2B (常染色体隐性)
1.733	TM6SF1	TM6SF1	跨膜 6 超家族成员 1
1.721	MYO1F	MYO1F	肌球蛋白 IF
1.691	CPR8; KIAA1254	CCPG1	细胞周期进行 1
1.688	LAB; NTAL; WSCR5; WBSCR5; HSPC046; WBSCR15	LAT2	T 细胞家族活化的连接物, 成员 2
1.687	CNAIP; FLJ40652;	NFAM1	具有 ITAM 基序 1 的 NFAT 活

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相对的经校正的表达	普通名称	基因符号	描述
	bK126B4.4		化蛋白
1.659	FVL; PCCF; 因子 V	F5	凝血因子 V (促凝血球蛋白原, 不稳定因子)
1.655	FLJ20273; DKFZp686F02235	FLJ20273	RNA-结合蛋白
1.647	NR4; CD213A1; IL-13Ra	IL13RA1	白细胞介素 13 受体, α 1
1.636	NCF; MGC3810; P40PHOX; SH3PXD4	NCF4	嗜中性粒细胞胞液的因子 4, 40kDa
1.635	p63; CLIMP-63; ERGIC-63; MGC99554	CKAP4	细胞骨架关联蛋白 4
1.611	SELR; SELX; MSRB1; HSPC270; MGC3344	SEPX1	含硒蛋白 X, 1
1.6	MD-2	LY96	淋巴细胞抗原 96
1.599	NPL1; c112; C1orf13; MGC61869; MGC149582	NPL	N-乙酰神经氨酸丙酮酸裂合酶(二氢吡啶二羧酸合成酶)
1.59	HAP; ASYIP; NSPL2; NSPLII; RTN3-A1	RTN3	reticulon 3
1.581	VMP1; DKFZP566I133	TMEM49	跨膜蛋白 49
1.567	HBP; HEBP	HEBP1	血红素结合蛋白 1
1.562	LAMPB; CD107b; LAMP-2C	LAMP2	溶酶体关联膜蛋白·2
1.559	C32; CKLF1; CKLF2; CKLF3; CKLF4; UCK-1; HSPC224	CKLF	趋化因子样因子
1.538		RASSF2	
1.532	SemE; SEMAE	SEMA3C	sema 功能域, 免疫球蛋白功

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相对的经校正的表达	普通名称	基因符号	描述
			能域 (Ig), 短的碱性功能域, 分泌的, (脑信号蛋白) 3C
1.53	ARAP3; DRAG1; FLJ21065	CENTD3	矢车菊苷, δ 3
1.516	HIG-1; C14orf75; FLJ36164; MGC135025; DKFZp434N0820	TDRD9	含有 tudor 功能域的 9
1.51	CAMKK; CAMKKB; KIAA0787; MGC15254	CAMKK2	钙/钙调蛋白-依赖的蛋白质激酶激酶 2, β
1.503	MEKK3; MAPKKK3	MAP3K3	有丝分裂活化的蛋白质激酶激酶激酶 3
1.488	AC; PHP; ASAH; PHP32; FLJ21558; FLJ22079	ASAH1	N-酰基鞘氨醇氨基水解酶 (酸性酰胺酶) 1
1.484	FCRN; α-链	FCGRT	IgG 的 Fc 片段, 受体, 转运物, α
1.479	MGC33054	SNX10	分选连接蛋白 10
1.474	HO68; VA68; VPP2; Vma1; ATP6A1; ATP6V1A1	ATP6V1A	ATPase, H+ 运输, 溶酶体 70kDa, V1 亚单位 A
1.466	MGST; GST12; MGST-I; MGC14525	MGST1	微粒体谷胱甘肽 S-转移酶 1
1.466	GAIP; RGSGAIP	RGS19	G-蛋白质信号转导 19 的调控剂
1.461	TKT1; FLJ34765	TKT	转羟乙醛酶 (Wernicke-Korsakoff 综合征)
1.449	S171	NUMB	numb 类似物 (果蝇)
1.448	FCHO2	FCHO2	FCH 功能域仅 2
1.444	LOC339745	LOC3397	待证实的蛋白质 LOC339745

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相对的经校正的表达	普通名称	基因符号	描述
		45	
1.443	CR3A; MO1A; CD11B; MAC-1; MAC1A; MGC117044	ITGAM	整合素, α M (补体组分 3 受体 3 亚单位)
1.442	D54; hD54; DKFZp686A1765	TPD52L2	瘤蛋白质 D52 样 2
1.432	MY014; KIAA0488; MGC20471; MGC126871; MGC126873	SNX27	分选连接蛋白家族成员 27
1.429	QK; Hqk; QK3; DKFZp586I0923	QKI	颤抖类似物, RNA 结合的 KH 功能域 (小鼠)
1.424	EVDB; D17S376	EVI2B	亲嗜性病毒整合位点 2B
1.424	PPT; CLN1; INCL	PPT1	棕榈酰-蛋白质硫酯酶 1 (蜡样质脂褐素沉积病, 二乙基溴乙酰胺 1, 婴儿的)
1.405	AOAH	AOAH	酰氧酰基水解酶 (嗜中性粒细胞)
1.404	MAY1; MGC49908; nPKC-δ	PRKCD	蛋白质激酶 C, δ
1.39	IMPA2	IMPA2	肌醇(myo)-1(或者 4)-单磷酸酯酶 2
1.382	ZYG11; FLJ13456	ZYG11B	zyg-11 类似物 B (线虫)
1.366	a3; Stv1; Vph1; Atp6i; OC116; OPTB1; TIRC7; ATP6N1C; ATP6V0A3; OC-116kDa	TCIRG1	T 细胞, 免疫调节剂 1, ATPase, H+运输, 溶酶体 V0 亚单位 A3
1.364	PGCP	PGCP	血浆谷氨酸羧肽酶
1.362	NNA1; KIAA1035; DKFZp686M20191	AGTPBP1	ATP/GTP 结合蛋白 1

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相对的经校正的表达	普通名称	基因符号	描述
1.355	TTG2; RBTN2; RHOM2; RBTNL1	LMO2	LIM 功能域仅 2 (菱形蛋白样 1)
1.344	CIP1; FLJ46905	SLC12A9	溶质载体家族 12 (钾/铝载体), 成员 9
1.34	ASRT5; IRAKM; IRAK-M	IRAK3	白细胞介素-1 受体关联的激酶 3
1.34	NEU; SIAL1	NEU1	唾液酸酶 1 (溶酶体唾液酸酶)
1.332	CRFB4; CRF2-4; D21S58; D21S66; CDW210B; IL-10R2	IL10RB	白细胞介素 10 受体, β
1.321	ASC; TMS1; CARD5; MGC10332	PYCARD	含有 PYD 和 CARD 功能域
1.31	KLHDC7C; KIAA0711	KBTBD11	含有 kelch 重复和 BTB (POZ) 功能域的 11
1.308	LTA4H	LTA4H	白细胞三烯 A4 水解酶
1.307	NR2B1; FLJ16020; FLJ16733; MGC102720	RXRA	类视黄醇 X 受体, α
1.303	JAM; KAT; JAM1; JAMA; JCAM; CD321; JAM-1; JAM-A; PAM-1	F11R	F11 受体
1.298	LH; LLH; PLOD	PLOD1	原胶原蛋白-赖氨酸 1, 2-酮戊二酸 5-双加氧酶 1
1.285	JTK8; FLJ26625	LYN	v-yes-1 Yamaguchi 肉瘤病毒性相关致癌基因类似物
1.281	MTX; MTXN	MTX1	metaxin 1
1.28	CGI-44	SQRDL	硫醌还原酶样 (酵母)
1.267	FLJ20424	C14orf94	染色体 14 开放阅读框 94
1.248	DCIR; LLIR; DDB27; CLECSF6;	CLEC4A	C-型凝集素功能域家族 4, 成员 A

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相对的经校正的表达	普通名称	基因符号	描述
	HDCGC13P		
1.238	EI; LEI; PI2; MNEI; M/NEI; ELANH2	SERPINB1	丝氨酸蛋白酶抑制蛋白肽酶抑制剂, 进化枝 B (卵白蛋白), 成员 1
1.234	3PK; MAPKAP3	MAPKAPK3	有丝分裂活化的蛋白质激酶活化的蛋白质激酶 3
1.227		ACSS2	
1.217	H2A.y; H2A/y; H2AFJ; mH2A1; H2AF12M; MACROH2A1.1; macroH2A1.2	H2AFY	H2A 组蛋白家族, 成员 Y
1.213	PP3856	NAPRT1	含有尼古丁酸磷酸核糖转移酶功能域的 1
1.212	ESP-2; HED-2	ZYX	斑联蛋白
1.179	SPC18; SPCS4A; SEC11L1; sid2895; 1810012E07Rik	SEC11L1	SEC11 类似物 A (酿酒酵母)
1.173	hEDTP; C3orf29; FLJ22405; FLJ90311	C3orf29	肌管蛋白相关的蛋白质 14
1.129	TGN38; TGN46; TGN48; TGN51; TTGN2; MGC14722	TGOLN2	反式高尔基网络蛋白 2

[0262] 与健康的对照相比，活性的 TB 组显示了 5281 个基因有差别地表达，如与潜伏性的组相比，仅显示了和对照相比的 3137 个有差别地表达的基因，可能反映了如细胞毒的模块中基因的过表达 / 表现显示的更为温和的（尽管清晰的）主动免疫响应。作为本发明的解释而非限制，这些结果很可能解释了与对照相比，在活性的 TB 患者中观察到的额外模块的变化这一现象，但是在相比对照的潜伏性的 TB 中不是如此。这些包括了在 M1.2（血小板，列于表 7A 中的基因）中的过达 / 表现，和在 M1.3（B 细胞，列于表 7B 中的基因）以及 M2.8（T 细胞，列于表 7H 中的基因）中低表达 / 表现的基因，可能期待后者，这是由于在 T 细胞对 M. tuberculosis 感染的响应中，T 细胞可能在感染的位点募集和 / 或在慢性感染期间受到抑制。在模块 M2.4 中的基因，下调表达的 / 表现的（列于表 7G 中的基因）包括编码核糖体蛋白家族成员的转录物（其表达在急性感染和脓毒疾中被改变 (Calvano, 2005 ; Thach, 2005)），以及在该模块中的基因已经显示在 SLE、肺移植的患者中以及 Streptococcus (S)

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pneumoniae 感染的人中低表达 (Chaussabel, Immunity,2005)。在活性的 TB 中,在模块 M3.1 中 (IFN- 可诱导的) 观察到最大集合的过量表达的基因 (90 个经检测的中的 60 个基因,表 7I),该基因的集合处于保护的 IFN-γ 的功能相符合,然而在此模块中的基因在潜伏性的 TB 患者中未有差别地表达,所述潜伏性的患者与对照相比控制了感染。在活性的 TB 中,基因在多个含有基因的模块 (M3.4、M3.6、M3.7、M3.8 和 M3.9,列于表 7L-7P 中的基因) 中低表达,所述基因不显示为一致的功能性模块,而是包括明显地不同集合的基因,并已经观察到在肺移植受体中低表达 (Chaussabel.,2008, Immunity)。

[0263] 根据对全血的转录分析并使用此模块图谱方法,可以将活性的 TB 患者从潜伏性的 TB 患者中区分。进一步地,在本研究中得到的活性的 TB 的模块图谱与其他的为不同的疾病创建的模块图谱的比较下,清楚的是,与 SLE、移植,黑色素瘤或者 S. pneumoniae 患者相比,活性的 TB 患者具有不同的总体转录谱 (图 9)(Chaussabel,2008, Immunity)。特定的模块如 M2.4 可能对于多种疾病是常见的,所述模块包括编码核糖体蛋白家族成员的转录物,其在活性的 TB、SLE、肺移植患者及感染了 S. pneumoniae 的人中是低表达的。然而,在其他的模块中的基因更不广泛地受影响,如 3.1(IFN- 可诱导的),其尽管在活性的 TB 中 (图 9) 和 SLE(Chaussabel,2008, Immunity) 中过表达,而在其他的疾病中不如此,尤其在 S. pneumoniae 中,此时其与对照相比,M3.1 中不显示有差别的基因表达。在关于在多个其他模块中的基因的过表达或者低表达,SLE 中的转录谱与活性的 TB 的不同。类似地,尽管在活性的 TB 及 S. pneumoniae 中观察到模块 M3.2 和 M3.3("炎症")、M1.2(血小板) 和 M1.5("髓的") 中的基因过表达,以及在模块 M3.4、5、6、7、8 和 9(非功能性的一致的模块) 中基因的低表达,这些疾病仍然能通过这种方法进行区别,这是由于与对照相比,模块 M2.2(中性粒细胞)、M2.3(红细胞)、M3.5(非功能上一致的模块) 中的基因在 S. pneumoniae 中过表达,而不在 TB 中有差别地受到影响。因此,通过保留数据的复杂性和重要性,然而将复杂的数据进行组织及减少其维度,可能将不同的感染性及炎症性疾病通过血液的转录谱区分开 (Chaussabel,2008, Immunity)。

[0264] 潜伏性和活性的 TB 患者的血液中,本发明鉴别出了谨慎的有差别的及相互的转录标签的数据集。具体地,活性的 TB 患者在功能性的 IFN- 可诱导的、炎症以及髓的模块中显示出基因的过表达 / 表现,其在另一个方面在潜伏性的 TB 中下调 / 低表现。活性的 TB 患者显示出免疫调节基因 PDL-1 和 PDL-2 的表达增加 / 过表现,其可能对 TB 中的免疫发病机理有贡献。来自潜伏性的 TB 患者血液显示出细胞毒性模块中基因的过表达 / 表现,其可能有助于保护性响应,该响应包括 M. tuberculosis 在这些患者中的感染,并可能为临床尝试中试验接种的效果提供生物标记物。我们相信我们初步研究的成功依赖于我们采用的严格的临床准则,为了支持潜在因素的归属进行的伴随免疫反应性研究,改进的 RNA 收集和分离的质量,先进的高通量全基因组微阵列平台,以及精密的数据挖掘工具,所述工具保持了基因表达的尺寸而具有可读的格式 (Chaussabel 等人提供)。该发现作为潜伏性的和活性的 TB 的诊断是有价值的,可能带来对下述的理解:免疫保护 (潜伏性的 TB) 相对于免疫发病 (活性的 TB) 的潜在机理 (这些转录区别所隐含的),以及用于保护的新型疗法的设计或者在活性的 TB 中免疫疗法的设计,从而利用抗分枝杆菌药物得到更快的治疗效果。

[0265] 预期在本说明书中讨论的,关于本发明的任意方法、试剂盒、试剂或者组合物的任意实施方案能够实现,反之亦真。进一步地,可以将本发明的组合物用于实现本发明的方

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法。

[0266] 应当理解的是在此描述的具体实施方案以说明的方式提供，而非作为本发明的限制。可能在不同的实施方案中采用本发明的主要特征，而不背离本发明的范围。本领域技术人员应认识到，或者能够确定使用不超过常规的试验，在此描述的特定过程的多个等价物。该等价物被认为在本发明的范围内，并由权利要求所覆盖。

[0267] 在本说明书中所提到的全部出版物和专利申请对于本发明相关领域的技术人员的技术水平而言是指示性的。将全部的出版物及专利申请在此结合作为参考，其引用的程度相当于指出各个单独的出版物或者专利申请特别地及单独地结合作为参考。

[0268] 词语“一个 (a)”或者“一个 (an)”的使用，当与术语“包括”在权利要求和 / 或说明书中一起使用时，其可能表示“一个”，然而其也可能与“一个或多个”，“至少一个”和“一个或者多于一个”的意思相同。虽然说明书支持仅指可选方案以及“和 / 或”的定义，术语“或者”在权利要求中的使用用于表示“和 / 或”，除非明确地指出其仅指可供选择的或者可选的方案是互相排斥的。在本申请中，术语“约“用于表示数值包括对于设备误差，所采用的以测定数值方法的固有的变化，或者在研究的对象中存在的变化。

[0269] 如本说明书和权利要求 (或多个权利要求) 中所使用的，词语“包括 (comprising)”(及包括的任意形式如“包括 (comprise)”和“包括 (comprises)”)，“具有 (having)”(及具有的任意形式，如“具有 (have)”和“具有 (has)”)，“包括 (including)”(及包括的任意形式如“包括 (includes)”和“包括 (include)”) 或者“含有 (containing)”(及含有的任意形式如“含有 (contains)”和“含有 (contain)”) 为包括的或者开放边界的，并不排除额外的、未列出的要素或者方法步骤。

[0270] 本文使用的术语“或其组合”指在该术语前所列出的个体的全部排列组合。例如，“A、B、B 或其组合”期待包括 A、B、C、AB、AC、BC 或者 ABC 的至少一种，以及如果顺序在特定环境下重要的话，还包括 BA、CA、CB、CBA、BCA、ACB、BAC 或者 CAB。继续此例子，包括一个或者多个要素或者术语的重复的组合也明确地包括在内，如 BB、AAA、MB、BBC、AAABCCCC、CBBAAA、CABABB 等等。技术人员理解典型地对于以任意组合的项目或者术语没有数目的限制，除非文字中明显表示的。

[0271] 本文公开并要求保护的全部的组合物和 / 或方法可能依照本公开在不过度的实验下制备和执行。尽管本发明的组合物和方法已经以优选的实施方式的形式进行了描述，对于本领域技术人员显而易见的是，可能对本文描述的组合物和 / 或方法进行变化，以及在所述方法的步骤或者步骤的顺序进行变化，而同时不悖离本发明的原理，精神以及范围。所有这样的对于本领域技术人员而言显而易见的类似替代和修改认为是在本发明由所附权利要求所限定的精神，范围及原理之内的。

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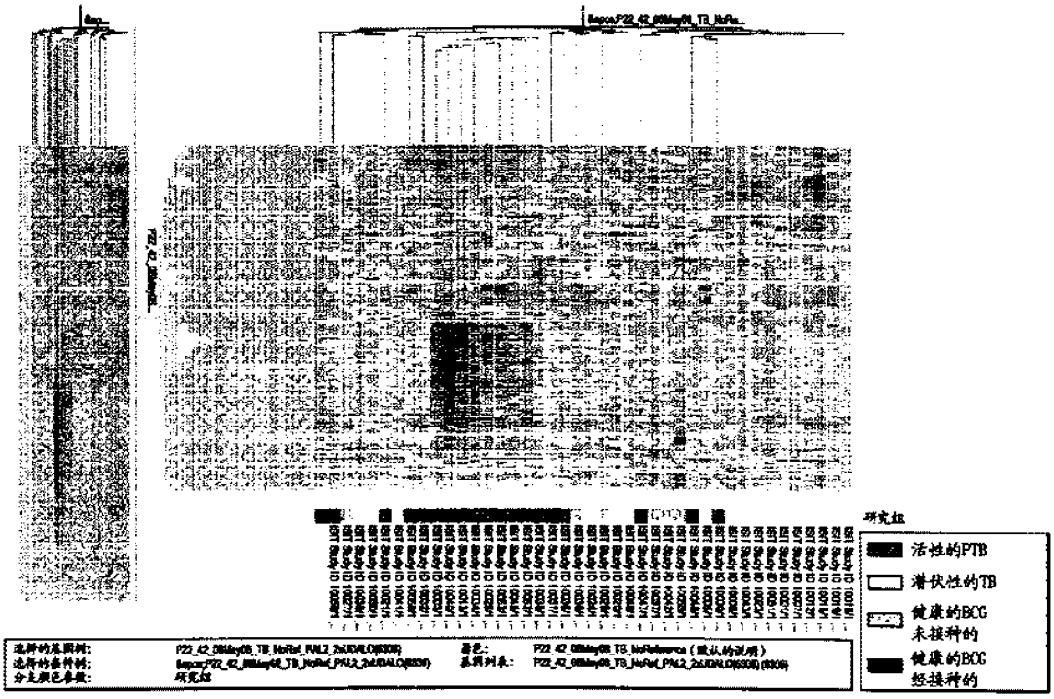


图 1

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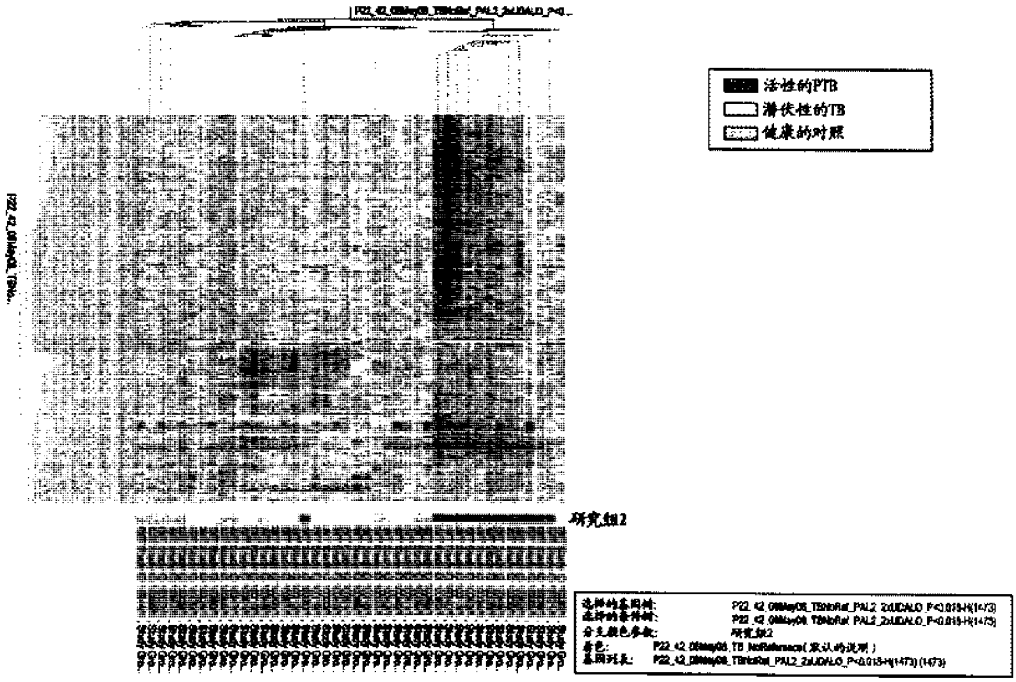


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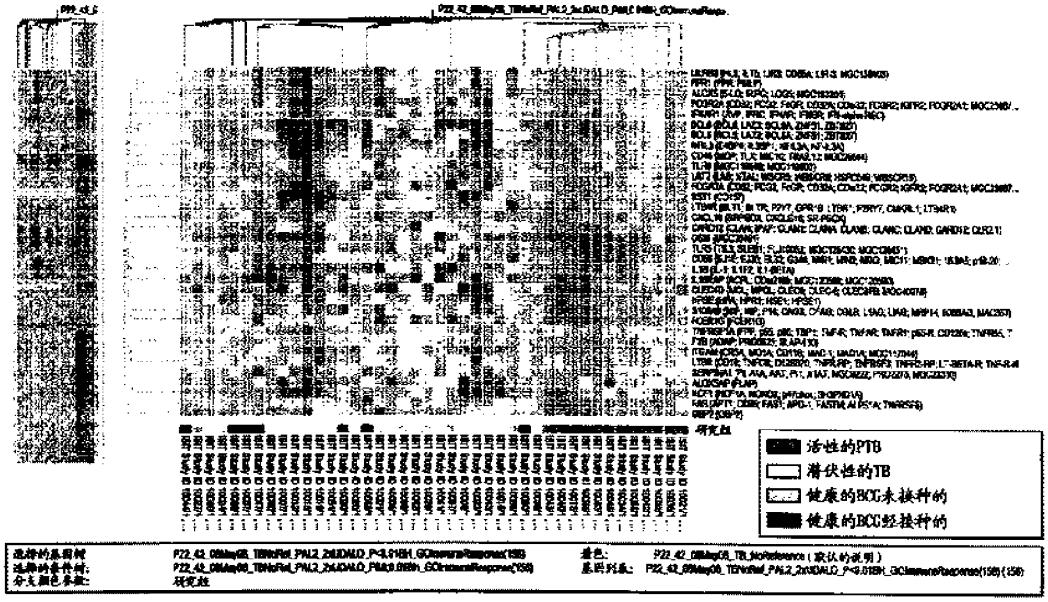


图 3A

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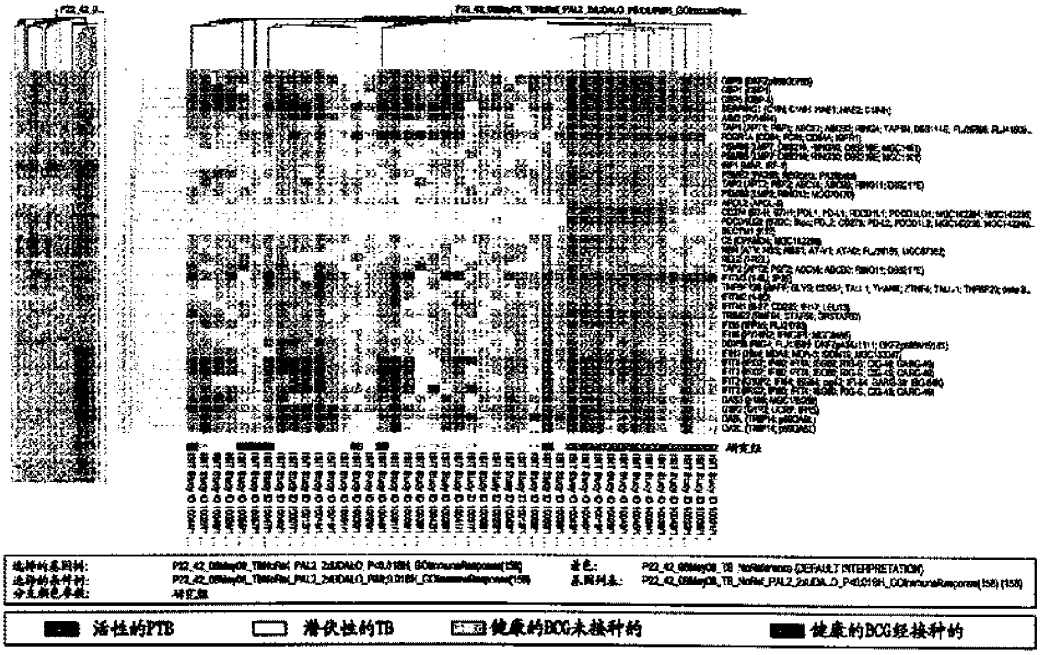


图 3B

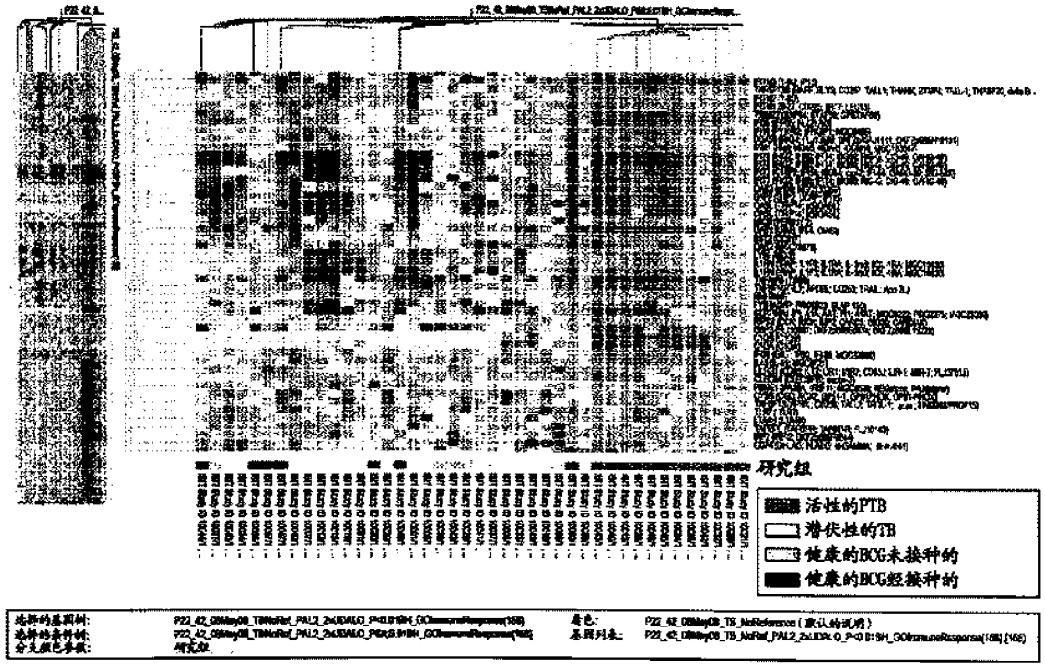


图 3C

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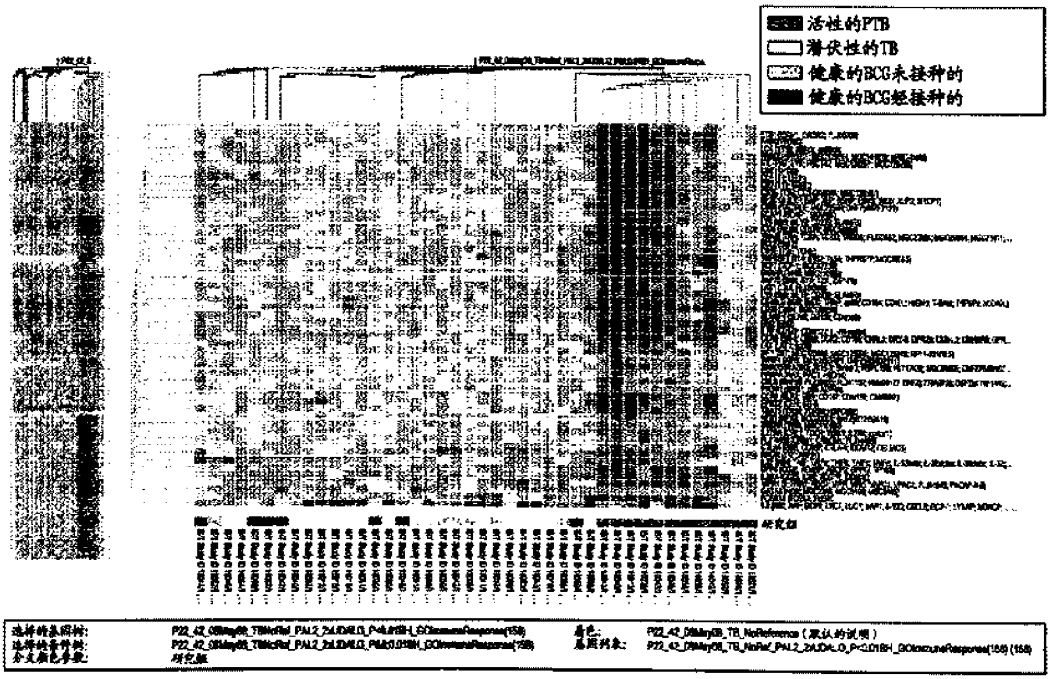


图 3D

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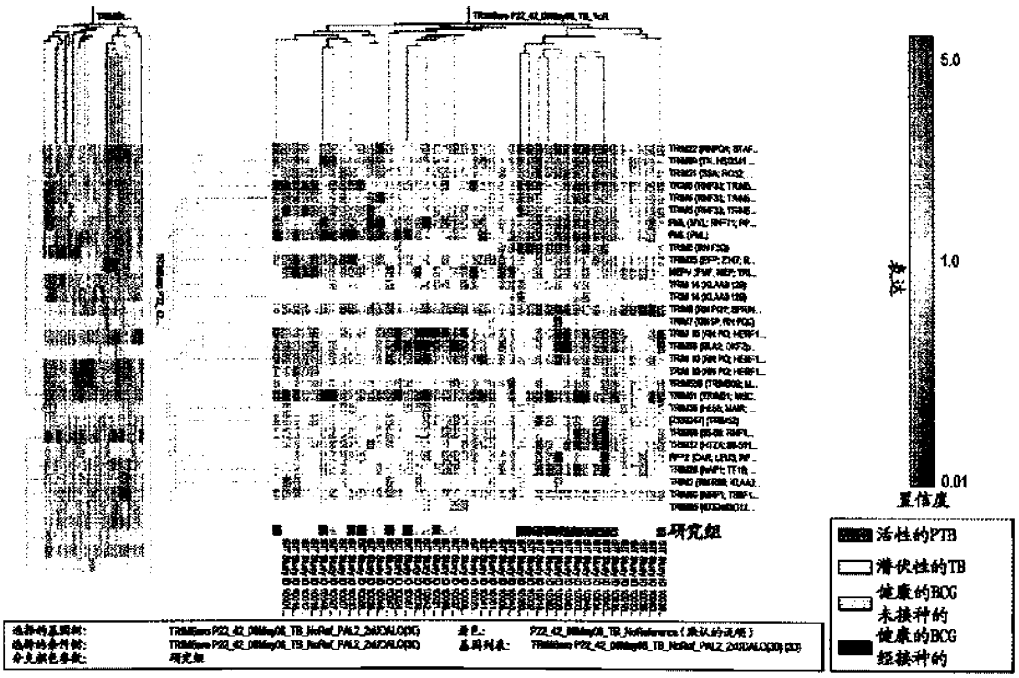


图 4

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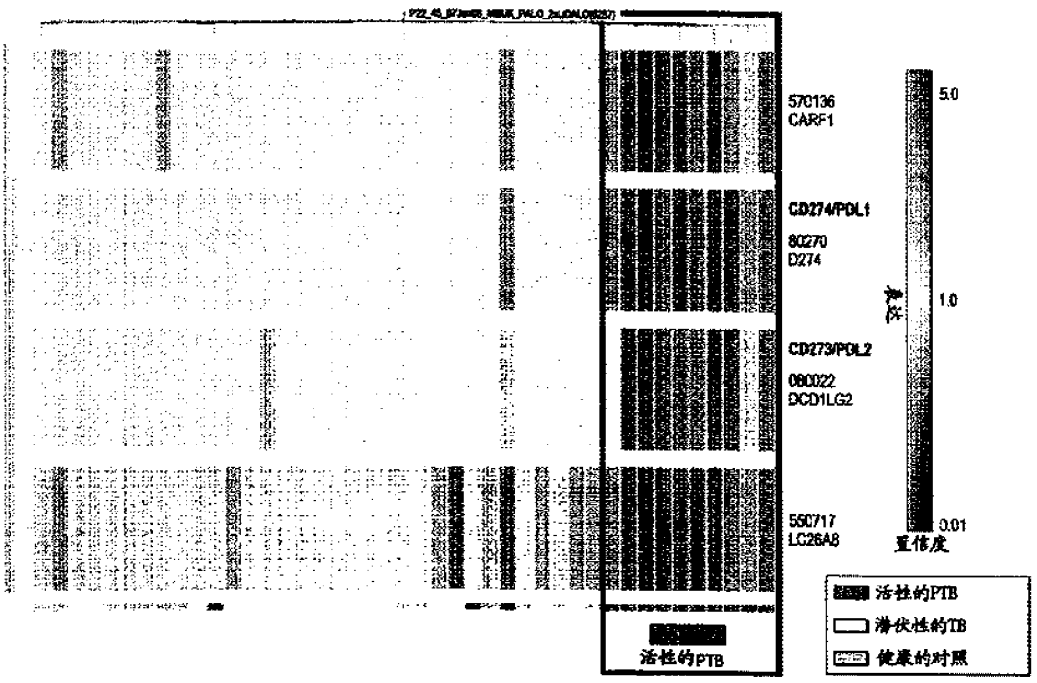


图 5A

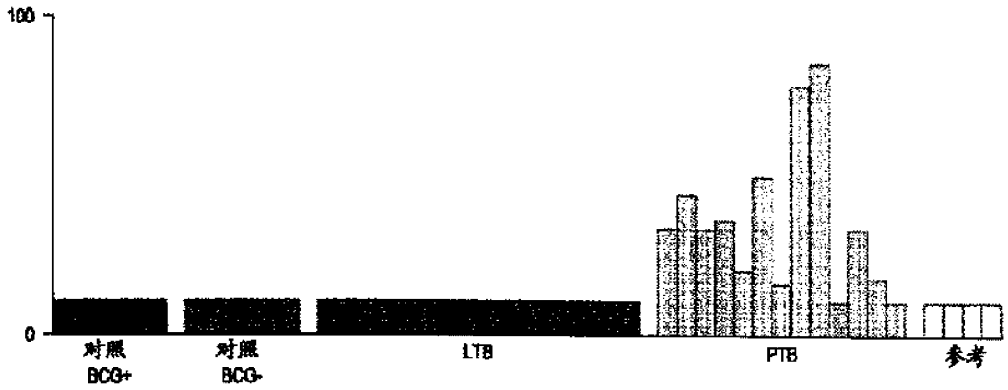


图 5B

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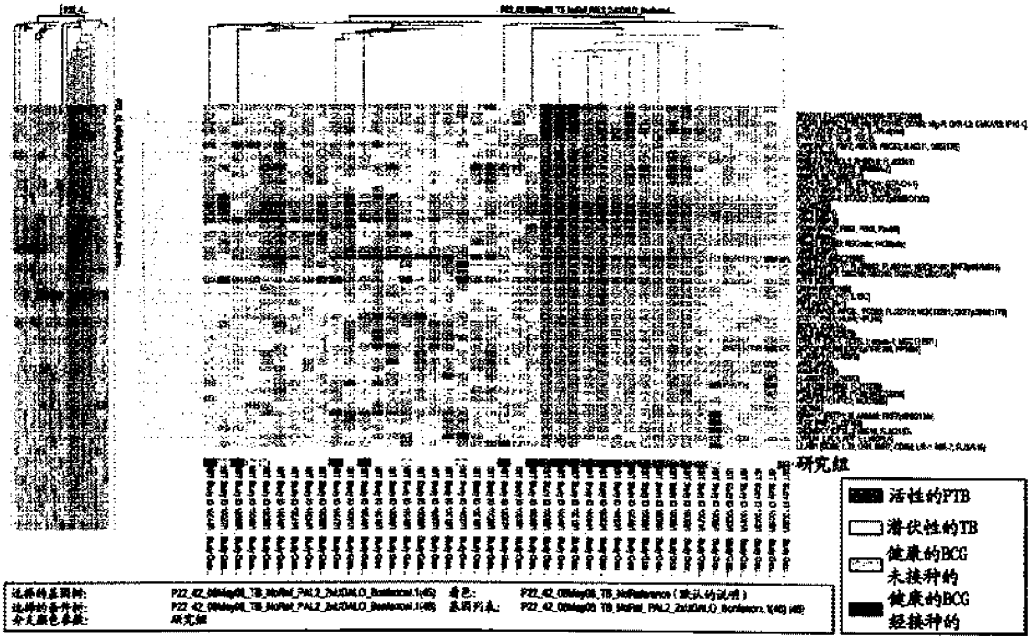


图 6

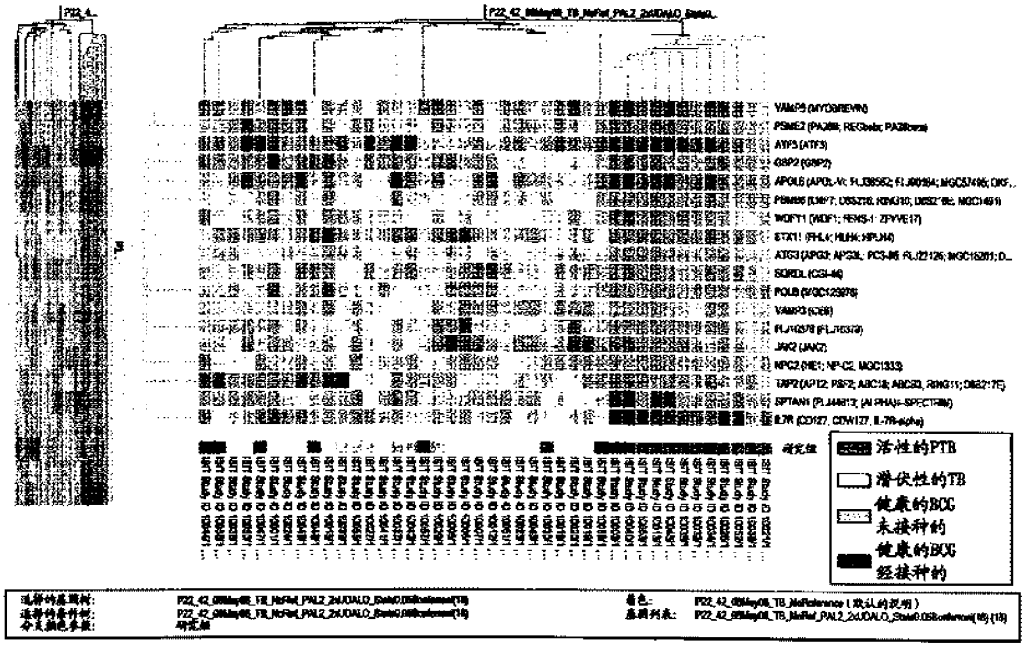


图 7

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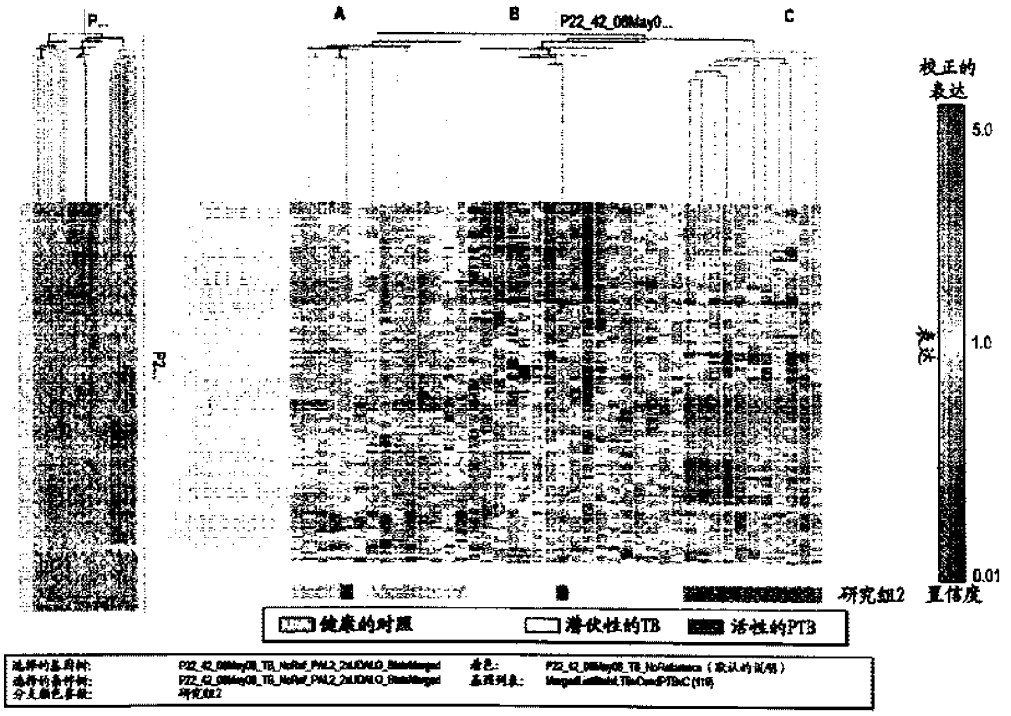


图 8A

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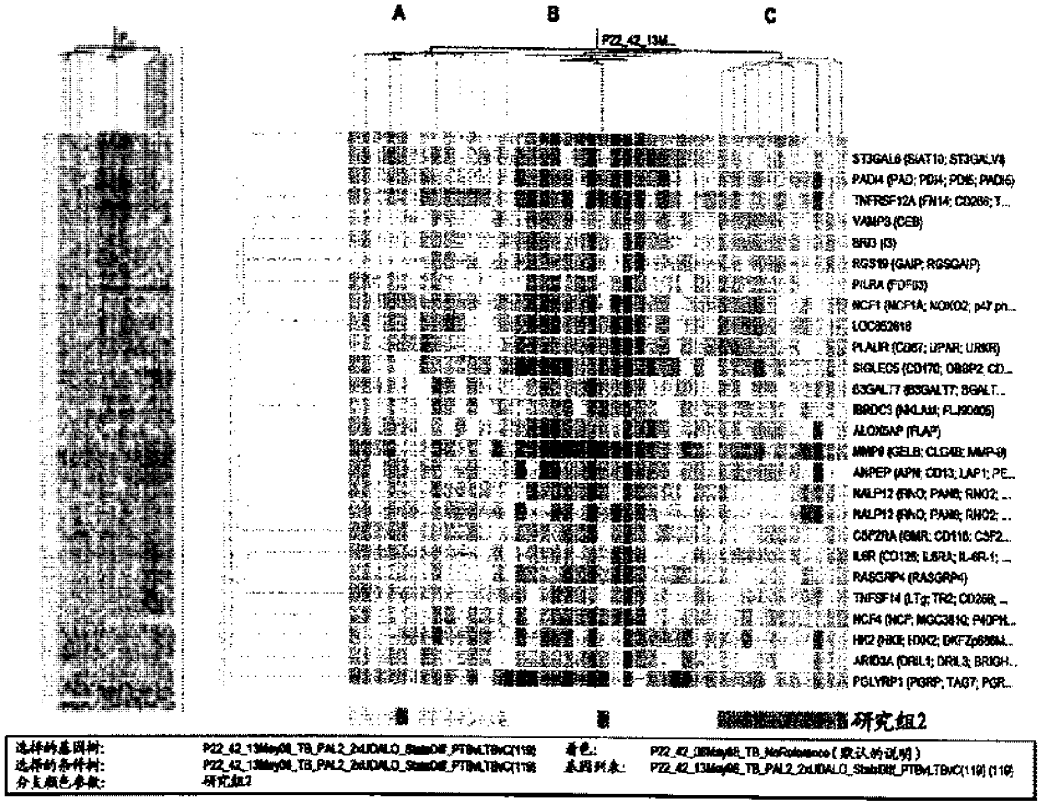


图 8B

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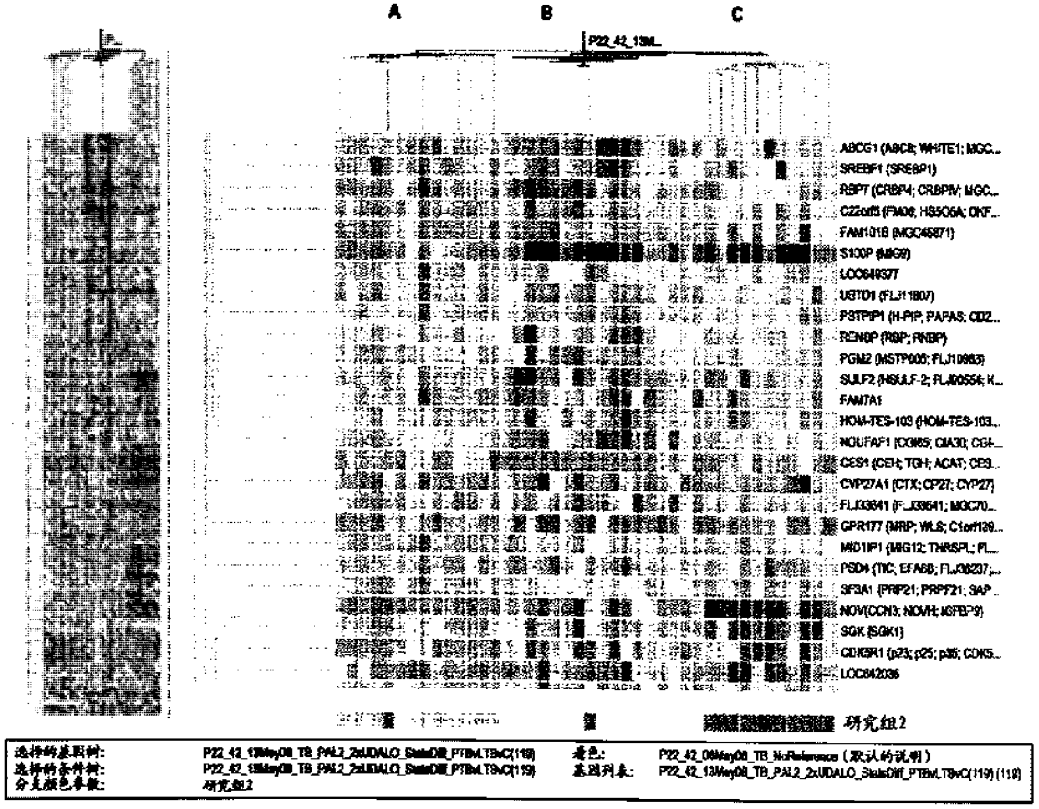


图 8C

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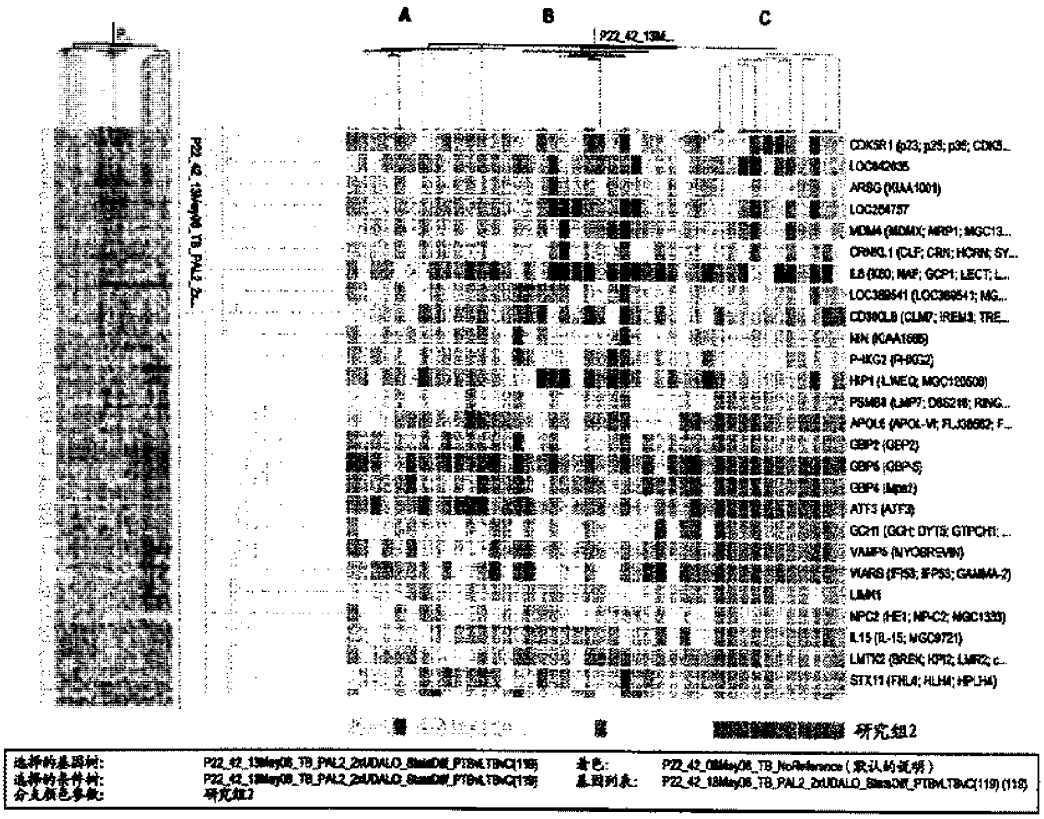


图 8D

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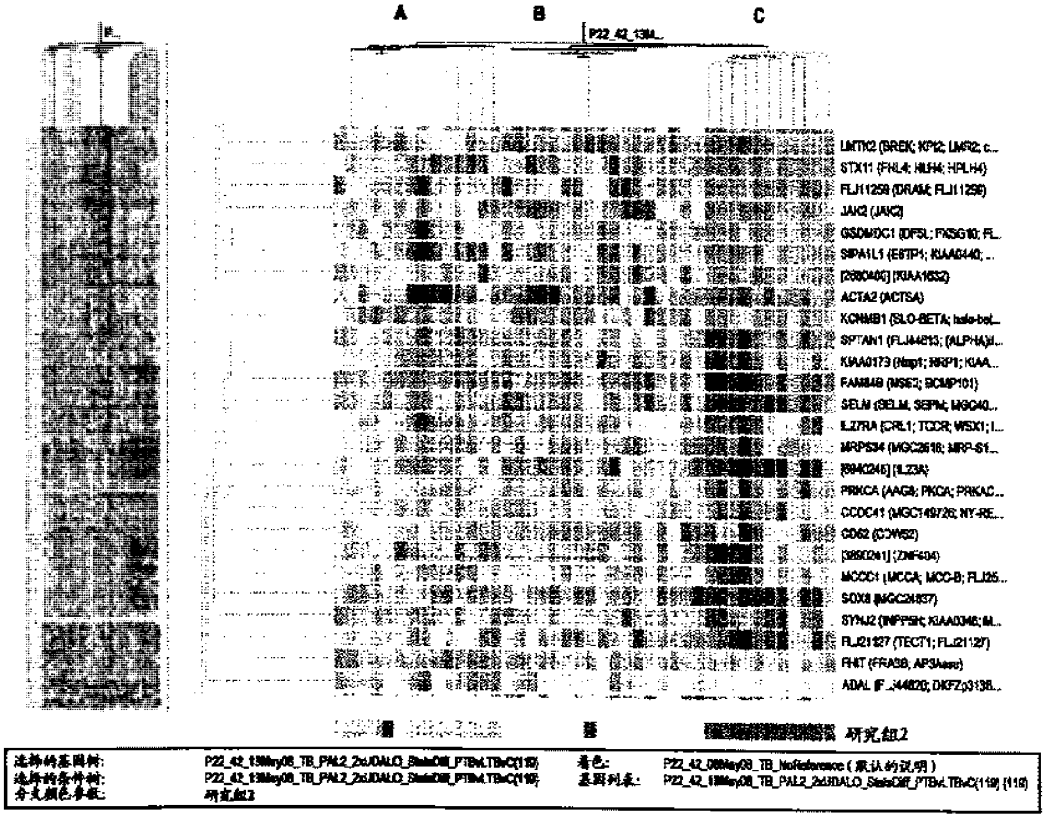


图 8E

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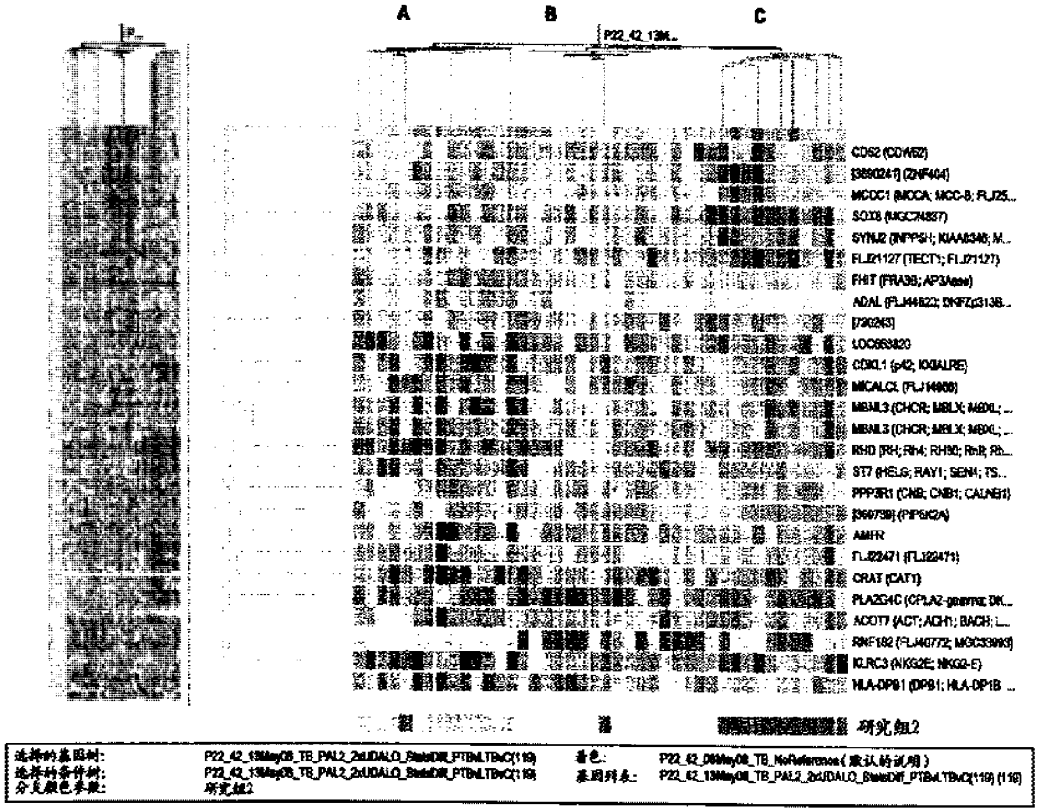


图 8F

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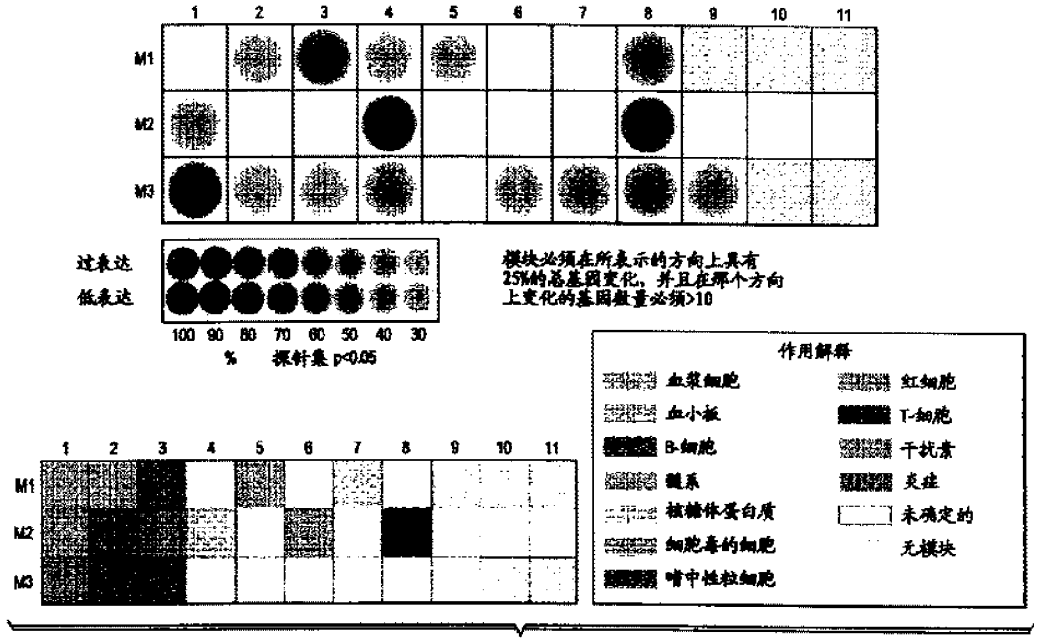


图 9

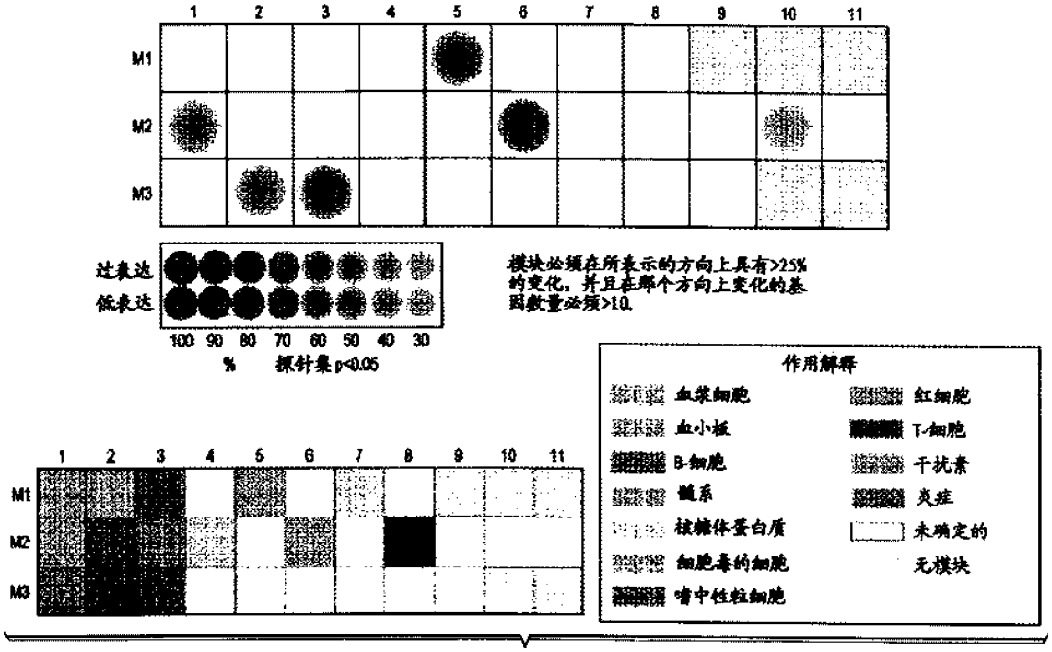


图 10

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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 18/352,112	Filing Date 07/13/2023	☐ To be Mailed

ENTITY: ☐ LARGE ☑ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $40 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $ 192 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	02/01/2024	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 30	Minus	** 30	= 0		x $40 =	0
	Independent (37 CFR 1.16(h))	* 3	Minus	*** 3	= 0		x $ 192 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/FREDERICK E BRISCOE/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
18/352,112	07/13/2023	Emil P. KARTALOV	88DG-373535-US

CONFIRMATION NO. 3145

30764
SHEPPARD, MULLIN, RICHTER & HAMPTON LLP
650 Town Center Drive, 10th Floor
Costa Mesa, CA 92626

PUBLICATION NOTICE



Date Mailed: 01/19/2024

Title:SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Publication No.US-2024-0018573-A1
Publication Date:01/18/2024

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The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

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IPR2025-01546
California Institute of Technology - Ex. 2026, Page 1309

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

30764 7590 01/05/2024

SHEPPARD, MULLIN, RICHTER & HAMPTON LLP
650 Town Center Drive, 10th Floor
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EXAMINER	
NEGIN, RUSSELL SCOTT	

ART UNIT	PAPER NUMBER
1672	

NOTIFICATION DATE	DELIVERY MODE
01/05/2024	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

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IPR2025-01546
California Institute of Technology - Ex. 2026, Page 1311

Office Action Summary	Application No. 18/352,112	Applicant(s) KARTALOV et al.	
	Examiner RUSSELL S NEGIN	Art Unit 1672	AIA (FITF) Status No

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

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

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

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1-30 is/are pending in the application.

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

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-30 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

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

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

Application Papers

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

11)☑ The drawing(s) filed on 13 July 2023 is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

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

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Comments

The present application is being examined under the pre-AIA first to invent provisions. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis (i.e., changing from AIA to pre-AIA) for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

Claims 1-30 are pending and examined in the instant Office action.

Information Disclosure Statements

The IDSs that have been filed have been considered.

Claim Rejections - 35 USC § 112(b) - Indefiniteness

The following is a quotation of 35 U.S.C. 112(b):
(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 3 and 19 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor (or for applications subject to pre-AIA 35 U.S.C. 112, the applicant), regards as the invention.

Claims 3 and 19 recite the equation $M=C*log_2(F+1)$ wherein M is the number of analytes, C is the number of channels, and F is the number of fluorophores. It is unclear as to the meets and bounds of M being an integer when F+1 is not a power of 2.

Claim 20 is not rejected under this statute because when F=3, F+1 is 4 (a power of 2).

Claim Rejections - 35 USC § 112(a) - Scope of Enablement

The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 3 and 19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification, while being enabling for providing a positive integer when F+1 is a power of 2, does not reasonably provide enablement for providing a positive integer when F+1 is not a power of 2. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

1. Claims 3 and 19 recite the equation $M=C*log_2(F+1)$ wherein M is the number of analytes, C is the number of channels, and F is the number of fluorophores. It is not understood as to how M is an integer when F+1 is not a power of 2.

Claim 20 is not rejected under this statute because when F=3, F+1 is 4 (a power of 2).

2. The specification is silent on how to determine M (a positive integer) when F+1 is not a power of 2.

3. The document of Han et al. [Nature Biotechnology, volume 19, 2001, pages 631-635; on IDS] teaches a system wherein each of the numbers of fluorophores, channels, and analytes are positive integers.

4. The claims recite an equation wherein the result (number of analytes) is not always a positive integer. The claims, specification, and prior art give no guidance on how to determine the number of analytes when F+1 is not a power of 2. Consequently, it is unpredictable as to determining the number of analytes (as positive integer) when F+1 is not a power of 2. Such unpredictability yields UNDUE EXPERIMENTATION.

In view of the above, it is the Examiner's position that with the insufficient guidance and working examples and in view of unpredictability and the state of art, one of skill in the art could not make and/or use the invention with the claimed breadth without an undue amount of experimentation.

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

35 U.S.C. 103 Rejection #1:

Claims 1-2, 5-14, 17-18, 20, 22-24, 27, and 29-30 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Han et al. [Nature Biotechnology, volume 19, 2001, pages 631-635; on IDS] in view of Sagner et al. [WO 2004/087950 A2; on IDS].

Claim 1 is drawn to a system comprising a sample chamber configured to house a sample and analyte-specific reagent mixtures of analyte-specific hybridization probes

and multiple fluorophores. The system comprises a multi-channel detector. This detector detects first, second, third, and fourth electromagnetic signals from first, second, third, and fourth wavelengths, respectively, from the sample chamber. The first, second, third, and fourth electromagnetic signals are generated by excitement of the first, second, third, and fourth fluorophores, respectively of the multiple fluorophores. The system comprises a processor controlled analyzer to receive, from the multi-channel detector, a cumulative signal based on the electromagnetic signals and apply a decoding matrix to the cumulative signal to unambiguously detect the presence or absence of each of M analytes by associating, for each analyte, a first value in a first component of the cumulative signal and a second value in a second component of the cumulative signal. Each first value is an intensity or range of intensities, and each second value is a wavelength or range of wavelengths. The second values comprise the first, second, third, and fourth wavelengths, and the determination is made without mass spectrometry or melting curve analysis. The analysis requires that M is greater than the number of second values used to encode the analytes, the multi-channel detector comprises C channels, and M and C are positive integers.

Claim 2 is further limiting wherein the analysis is extended to fifth and sixth electromagnetic signals from fifth and sixth fluorophores.

Claim 20 is drawn to similar subject matter as claim 2, except claim 20 is drawn to a reaction mixture.

Claim 30 is drawn to similar subject matter as claim 20, except claim 30 is drawn to a kit.

The document of Han et al. studies quantum-dot-tagged microbeads for multiplexes optical coding of biomolecules [title]. Figure 1 on page 632 of Han et al. teaches that there are ten distinguishable colors of quantum dots that emit light at the ten listed wavelengths. Figures 5 and 6 of Han et al. use microbeads and hybridization to detect the presence of three of the quantum dot fluorophores. The spectral data of Figures 5 and 6 of Han et al. illustrates a matrix of intensity values of the fluorophores as a function of wavelength of light. The number of analytes in Figure 1 of Han et al. (10) is greater than the numbers of second values in each of Figures 5 and 6 Han et al. (3).

Han et al. does not teach all of the channel/apparatus limitations of the claims.

The document of Sagner et al. studies an improved system for multicolor real-time PCR [title]. The cover figure of Sagner et al. illustrates the detection unit with six different detectors that measure signals at six different channels of wavelength. Page 9 of Sagner et al. lists the fluorophores. Page 10 of Sagner et al. lists wavelengths analyzed.

With regard to claim 5, Figures 5-6 of Han et al. illustrates blue, green, and red fluorophores.

With regard to claims 6 and 22, page 9 of Sagner et al. teaches use of the cyanine fluorophores.

With regard to claims 7-14, 17-18, 23-24, and 27, Figures 5-6 of Han et al. illustrates blue, green, and red fluorophores attached to hybridization probes. The spectral data of Figures 5-6 of Han et al. illustrate plots of the first and second values. Figure 1 of Han et al. lists the wavelengths of all ten possible quantum dots. The cover figure of Sagner et al. lists the wavelengths of each of the six channels/filters/photodetectors where blue, green, and red are a subset of the colors that are detected.

With regard to claim 29, Figures 5-6 of Han et al. illustrate different hybridization probes at different concentrations in a mixture.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the quantum dot analysis of Han et al. by use of the apparatus to analyze fluorophores of Sagner et al. wherein the motivation would have been that Sagner et al. teaches additional equipment to facilitate analysis of spectral data [cover figure of Sagner et al.]. There would have been a reasonable expectation of success to combine Han et al. and Sagner et al. because both studies are analogously applicable to analyzing spectral data relates to labeled biomolecules, either by quantum dots or fluorophores.

35 U.S.C. 103 Rejection #2:

Claims 15-16, 21, 25-26, and 28 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Han et al. in view of Sagner et al. as applied to claims 1-2,

5-14, 17-18, 20, 22-24, 27, and 29-30 above, in further view of Vogelstein et al. [PNAS, volume 96, 1999, pages 9236-9241; on IDS].

Claims 15 and 21 are further limiting wherein the sample comprises a droplet. Claim 16 is further limiting wherein the droplets are housed in a dynamic array.

Claims 25-26 constrain ratios of probe concentrations and hybridization procedures.

Claim 28 is further limiting wherein a probe comprises a quencher.

Han et al. and Sagner et al. make obvious analyze of labeled biomolecules using a multichannel apparatus, as discussed above.

Han et al. and Sagner et al. do not teach the droplet, array, ratio of concentrations, quenchers, and hybridization procedures in the instantly rejected claims.

The document of Vogelstein et al. studies digital PCR [title]. Figure 1 on page 9237 of Vogelstein et al. illustrates droplets in a dynamic array and probe attached to a quencher. The Methods section on pages 9236-9237 of Vogelstein et al. teaches the hybridization procedures. A subset of the cells in Figure 5 on pages 9240 of Vogelstein et al. illustrates the recited ratios of hybridization probes.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the quantum dot analysis of Han et al. and the apparatus to analyze fluorophores of Sagner et al. by use of the digital PCR of Vogelstein et al. wherein the motivation would have been that Sagner et al. teaches additional empirical techniques to facilitate analysis of experimental data [Figure 1 of Vogelstein et al.]. There would have been a reasonable expectation of success to combine Han et al.,

Sagner et al., and Vogelstein et al. because all three studies are analogously applicable to analyzing spectral data relates to labeled biomolecules, either by quantum dots or fluorophores.

Double Patenting

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

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file

provisions of the AIA as explained in MPEP § 2159. See MPEP § 2146 *et seq.* for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The filing of a terminal disclaimer by itself is not a complete reply to a nonstatutory double patenting (NSDP) rejection. A complete reply requires that the terminal disclaimer be accompanied by a reply requesting reconsideration of the prior Office action. Even where the NSDP rejection is provisional the reply must be complete. See MPEP § 804, subsection I.B.1. For a reply to a non-final Office action, see 37 CFR 1.111(a). For a reply to final Office action, see 37 CFR 1.113(c). A request for reconsideration while not provided for in 37 CFR 1.113(c) may be filed after final for consideration. See MPEP §§ 706.07(e) and 714.13.

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

Double Patenting Rejection #1:

Claim 19 is rejected on the ground of nonstatutory double patenting as being unpatentable over claim 1 of U.S. Patent No. 8,838,394 B2 [on IDS] in view of Sagner et al.

Both claims are drawn to detecting the presence and absence of analytes using signals from fluorophores. However, the claim of '394 does not teach all of the apparatus limitations of the instantly rejected claim.

The document of Sagner et al. studies an improved system for multicolor real-time PCR [title]. The cover figure of Sagner et al. illustrates the detection unit with six different detectors that measure signals at six different channels of wavelength.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the fluorophore analysis of the claim of '394 by use of the apparatus to analyze fluorophores of Sagner et al. wherein the motivation would have been that Sagner et al. teaches additional equipment to facilitate analysis of spectral data [cover figure of Sagner et al.]. There would have been a reasonable expectation of success to combine the claim of '394 and Sagner et al. because both studies are analogously applicable to analyzing spectral data relates to fluorophore labeled biomolecules.

Double Patenting Rejection #2:

Claim [1 or 20] is rejected on the ground of nonstatutory double patenting as being unpatentable over claim 1 of U.S. Patent No. 10,068,051 B2 [on IDS] in view of Sagner et al.

Both sets of claims are drawn to detecting the presence and absence of analytes using signals from fluorophores. However, the claim of '051 does not teach all of the apparatus limitations of the instantly rejected claims.

The document of Sagner et al. studies an improved system for multicolor real-time PCR [title]. The cover figure of Sagner et al. illustrates the detection unit with six different detectors that measure signals at six different channels of wavelength.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the fluorophore analysis of the claim of '051 by use of the apparatus to analyze fluorophores of Sagner et al. wherein the motivation would have been that Sagner et al. teaches additional equipment to facilitate analysis of spectral data [cover figure of Sagner et al.]. There would have been a reasonable expectation of success to combine the claim of '051 and Sagner et al. because both studies are analogously applicable to analyzing spectral data relates to fluorophore labeled biomolecules.

Double Patenting Rejection #3:

Claims [1, 15, 20, or 21] are rejected on the ground of nonstatutory double patenting as being unpatentable over claim 1 of U.S. Patent No. 10,770,170 B2 [on IDS] in view of Sagner et al.

Both sets of claims are drawn to detecting the presence and absence of analytes using signals from fluorophores. However, the claim of '170 does not teach all of the apparatus limitations of the instantly rejected claims.

The document of Sagner et al. studies an improved system for multicolor real-time PCR [title]. The cover figure of Sagner et al. illustrates the detection unit with six different detectors that measure signals at six different channels of wavelength.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the fluorophore analysis of the claim of '170 by use of the apparatus to analyze fluorophores of Sagner et al. wherein the motivation would have been that Sagner et al. teaches additional equipment to facilitate analysis of spectral data [cover figure of Sagner et al.]. There would have been a reasonable expectation of success to combine the claim of '170 and Sagner et al. because both studies are analogously applicable to analyzing spectral data relates to fluorophore labeled biomolecules.

Double Patenting Rejection #4:

Claim [1 or 20] is provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claim 22 of copending Application No. 16/937,464 in view of Sagner et al.

Application '464 has been issued.

Both sets of claims are analogously drawn to detecting the presence and absence of analytes using signals from fluorophores. However, the claim of '464 does not teach all of the apparatus limitations of the instantly rejected claims.

The document of Sagner et al. studies an improved system for multicolor real-time PCR [title]. The cover figure of Sagner et al. illustrates the detection unit with six different detectors that measure signals at six different channels of wavelength.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the fluorophore analysis of the claim of '464 by use of the apparatus to analyze fluorophores of Sagner et al. wherein the motivation would have been that Sagner et al. teaches additional equipment to facilitate analysis of spectral data [cover figure of Sagner et al.]. There would have been a reasonable expectation of success to combine the claim of '464 and Sagner et al. because both studies are analogously applicable to analyzing spectral data relates to fluorophore labeled biomolecules.

Allowable Subject Matter

Claim 4 is objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

The prior art does not teach the equation in the claim, and more specifically, the prior art does not teach the equation with the specific values of M, C, and F.

E-mail Communications Authorization

Per updated USPTO Internet usage policies, Applicant and/or applicant's representative is encouraged to authorize the USPTO examiner to discuss any subject matter concerning the above application via Internet e-mail communications. See MPEP 502.03. To approve such communications, Applicant must provide written authorization for e-mail communication by submitting the following statement via EFS-Web (using PTO/SB/439) or Central Fax (571-273-8300):

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Conclusion

No claim is allowed.

Any inquiry concerning this communication or earlier communications from the Examiner should be directed to Russell Negin, whose telephone number is (571) 272-1083. This Examiner can normally be reached from Monday through Thursday from 8 am to 3 pm and variable hours on Fridays.

If attempts to reach the Examiner by telephone are unsuccessful, the Examiner's Supervisor, Larry Riggs, Supervisory Patent Examiner, can be reached at (571) 270-3062.

Information regarding the status of the application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for

published applications may be obtained from either Private PAIR or Public PAIR.

Status information for unpublished applications is available through Private PAIR only.

/RUSSELL S NEGIN/
Primary Examiner, Art Unit 1672
31 December 2023

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/352,112	KARTALOV et al.
	Examiner	Art Unit
	RUSSELL S NEGIN	1672

CPC - Searched*		
Symbol	Date	Examiner
C12Q1/6825 or C12Q1/68 or C12Q1/6851 or G01N21/6486 or G16B5/00 or G16B25/00 or G16B25/20 or G16B40/10 or G06F17/10 or G06F17/11 or G16B40/00	12/30/2023	RSN

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
see attached search history printouts	12/30/2023	RSN
PE2E Search prior art search	12/30/2023	RSN
PE2E Search inventor search on all inventors	12/30/2023	RSN
PDP inventor search		
PE2E Search CPC code search with modified terms	12/30/2023	RSN

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/RUSSELL S NEGIN/ Primary Examiner, Art Unit 1672	

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/RUSSELL S NEGIN/ Primary Examiner, Art Unit 1672	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 1 of 27

Complete if Known	
Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

U. S. PATENT DOCUMENTS

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

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Sheet 2 of 27

Complete if Known	
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Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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

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Sheet 3 of 27

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First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

U. S. PATENT DOCUMENTS

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

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Sheet 4 of 27

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Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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

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Examiner Signature	/RUSSELL S NEGIN/	Date Considered	12/30/2023

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Sheet 5 of 27

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First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
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	081	US-20050250146	11-10-2005	MCMILLAN; William A.	
	082	US-20050260640	11-24-2005	ANDERSEN; Mark R. et al.	
	083	US-20060216708	09-28-2006	VENEMA; Fokke	
	084	US-20070072211	03-29-2007	NEWTON; Nick et al.	
	085	US-20070178485	08-02-2007	EL-DEIRY; Wafik S. et al.	
	086	US-20070231824	10-04-2007	CHEE; Mark S. et al.	
	087	US-20080050737	02-28-2008	ARIELI; Boaz et al.	
	088	US-20080069733	03-20-2008	MALTEZOS; George et al.	
	089	US-20080096767	04-24-2008	KOHN; Heinz G.	
	090	US-20080124705	05-29-2008	KRAMER; Fred R.	
	091	US-20090042735	02-12-2009	BLAIR; Steven M. et al.	
	092	US-20090048785	02-19-2009	KATZIR; NIR et al.	
	093	US-20090062129	03-05-2009	MCKERNAN; Kevin [Us] et al.	
	094	US-20100015607	01-21-2010	GEISS; Gary K. et al.	
	095	US-20100041092	02-18-2010	LIN; Michael Z. et al.	
	096	US-20100047924	02-25-2010	WEBSTER; Philippa J. et al.	
	097	US-20100112710	05-06-2010	GEISS; Gary K. et al.	
	098	US-20100120043	05-13-2010	SOOD; Anup et al.	
	099	US-20100151443	06-17-2010	XIANG; Qing et al.	
	100	US-20100159447	06-24-2010	LI; BIN et al.	
	101	US-20100210472	08-19-2010	EMPEDOCLES; Stephen A. et al.	

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First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
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	102	US-20100233686	09-16-2010	HIGUCHI; Russell et al.	
	103	US-20100248257	09-30-2010	JACOBSEN; Kivin et al.	
	104	US-20100261026	10-14-2010	FERREE; Sean M. et al.	
	105	US-20100267064	10-21-2010	KARTALOV; Emil P. et al.	
	106	US-20100273173	10-28-2010	HIRAI; Mitsuharu et al.	
	107	US-20100324834	12-23-2010	TREPTOW; Rainer [De] et al.	
	108	US-20110104684	05-05-2011	HOOPER; Dennis G.	
	109	US-20110151459	06-23-2011	ROTHMANN; Thomas et al.	
	110	US-20110151550	06-23-2011	SAGNER; Gregor et al.	
	111	US-20110171658	07-14-2011	CARRICK; James M.	
	112	US-20110207623	08-25-2011	DIMITROV; Krassen et al.	
	113	US-20110223602	09-15-2011	WHITMAN; Douglas F. [Us] et al.	
	114	US-20110237459	09-29-2011	NOVA; Michael P. et al.	
	115	US-20120003646	01-05-2012	JOO; Sunghae A. et al.	
	116	US-20120040349	02-16-2012	VON; Lode Piia et al.	
	117	US-20120040352	02-16-2012	WANGH; Lawrence J. [Us] et al.	
	118	US-20120045756	02-23-2012	ROTHMANN; Thomas et al.	
	119	US-20120077195	03-29-2012	LI; Qingge et al.	
	120	US-20120101740	04-26-2012	ORPANA; Arto [Se] et al.	
	121	US-20120122704	05-17-2012	ATCHLEY; Daniel H. et al.	

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Sheet 7 of 27

Complete if Known	
Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

U. S. PATENT DOCUMENTS

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

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Complete if Known	
Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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

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Sheet 9 of 27

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First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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

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Sheet 10 of 27

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First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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

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First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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

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Sheet 12 of 27

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First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 13 of 27

Complete if Known	
Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	041	WO-2012135340-A2	10-04-2012	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	042	WO-2012135340-A3	12-20-2012	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	043	WO-2013096851-A1	06-27-2013	HARVARD COLLEGE [US]		☐
	044	WO-2013116780-A1	08-08-2013	CALIFORNIA INST OF TECHN [US], et al.		☐
	045	WO-2014022827-A1	02-06-2014	CALIFORNIA INST OF TECHN [US]		☐
	046	WO-2014116884-A1	07-31-2014	CALIFORNIA INST OF TECHN [US]		☐
	047	WO-2015147370-A1	10-01-2015	SEEGENE INC [KR]		☐
	048	WO-2016154600-A1	09-29-2016	MASSACHUSETT S GEN HOSPITAL [US]		☐
	049	WO-2017139354-A1	08-17-2017	UNIV MICHIGAN REGENTS [US]		☐
	050	WO-2017173035-A1	10-05-2017	CHROMACODE INC [US]		☐

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	051	WO-2017218777-A1	12-21-2017	CALIFORNIA INST OF TECHN [US]		☐
	052	WO-2018081178-A1	05-03-2018	TWO PORE GUYS INC [US]		☐
	053	WO-2019006023-A1	01-03-2019	CHROMACODE INC [US]		☐
	054	WO-2019079204-A1	04-25-2019	CHROMACODE INC [US]		☐
	055	WO-2019169043-A1	09-06-2019	CHROMACODE INC [US]		☐
	056	WO-9746714-A1	12-11-1997	UNIV UTAH RES FOUND [US], et al.		☐
	057	WO-99/52708	10-21-1999	LUMINEX CORP [US]		☐
	058	WO-9919515-A1	04-22-1999	LUMINEX CORP [US]		☐

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

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

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	020	Extended European Search Report for Patent Application No. EP13824744.0 dated February 26, 2016.	☐
	021	FODOR, et al. Multiplexed biochemical assays with biological chips. Nature. 1993 Aug 5;364(6437):555-6.	☐
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	041	LAO, et al. Multiplexing RT-PCR for the detection of multiple miRNA species in small samples. Biochem Biophys Res Commun. 2006 Apr 28;343(1):85-9. Epub 2006 Feb 28.	☐
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 20 of 27

Complete if Known	
Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

NON-PATENT LITERATURE DOCUMENTS

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	050	MORGAN et al., A commercial line probe assay for the rapid detection of rifampicin resistance in Mycobacterium tuberculosis: a systematic review and meta-analysis. BMC Infectious Diseases. 5:62 doi:10.1186/1471-2334-5-62 (2005).	☐
	051	MORRISON, et al. Two-color ratio-coding of chromosome targets in fluorescence in situ hybridization: quantitative analysis and reproducibility. Cytometry. 1997 Apr 1;27(4):314-26.	☐
	052	MYERS et al., A Handheld Point-of-Care Genomic Diagnostic System. PLoS One 8(8): e70266; pages 1-9 (2013).	☐
	053	NAVARRO et al., Real-time PCR detection chemistry. Clin Chim Acta. 439:231-250 (2015).	☐
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	055	OLIVEIRA, et al. Multiplex PCR strategy for rapid identification of structural types and variants of the mec element in methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother. 2002 Jul;46(7):2155-61.	☐
	056	OU, et al. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science. 1988 Jan 15;239(4837):295-7.	☐
	057	PATON, et al. Detection and characterization of Shiga toxigenic Escherichia coli by using multiplex PCR assays for stx1, stx2, eaeA, enterohemorrhagic E. coli hlyA, rfbO111, and rfbO157. J Clin Microbiol. 1998 Feb;36(2):598-602.	☐
	058	PATTERSON, et al. Detection of HIV-1 DNA and messenger RNA in individual cells by PCR-driven in situ hybridization and flow cytometry. Science. 1993 May 14;260(5110):976-9.	☐

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

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First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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	072	PCT/US2019/035129 Invitation to Pay Additional Fees dated August 19, 2019.	☐
	073	PCT/US2019/040392 International Preliminary Report on Patentability dated January 05, 2021.	☐
	074	PCT/US2019/041239 International Preliminary Report on Patentability dated January 21, 2021.	☐
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	080	RAJAGOPAL et al. Supercolor Coding Methods for Large-Scale Multiplexing of Biochemical Assays. Analytical Chemistry 85:7629-7636 (2013).	☐
	081	RAJAGOPAL et al., Significant Expansion of Real-Time PCR Multiplexing with Traditional Chemistries using Amplitude Modulation. Scientific Reports. 9(1): 1053 (2019). .	☐

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Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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	082	Rickert et al., "Multiplexed Real-Time PCR Using Universal Reporters," Clin. Chem., 50(9):1680- 1683, 2004.	☐
	083	ROSENSTRAUS, et al. An internal control for routine diagnostic PCR: design, properties, and effect on clinical performance. J Clin Microbiol. 1998 Jan;36(1):191-7.	☐
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	085	SAIKI, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, 239(4839):487-491 (January 29, 1988).	☐
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	089	SPEICHER, et al. Karyotyping human chromosomes by combinatorial multi-fluor FISH. Nat Genet. 1996 Apr;12(4):368-75.	☐
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	091	TIRASOPHON, et al. A novel detection of a single Plasmodium falciparum in infected blood. Biochem Biophys Res Commun. 1991 Feb 28;175(1):179-84.	☐

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Sheet 24 of 27

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First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
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	092	TYAGI, et al. Multicolor molecular beacons for allele discrimination. Nat Biotechnol. 1998 Jan;16(1):49-53.	☐
	093	TYAGI, et al. Wavelength-shifting molecular beacons. Nat Biotechnol. 2000 Nov;18(11):1191-6.	☐
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	095	URDEA, et al. Requirements for high impact diagnostics in the developing world. Nature. 2006 Nov 23;444 Suppl 1:73-9.	☐
	096	U.S. Application No. 13/756,760 Notice of allowance dated June 2, 2014.	☐
	097	U.S. Application No. 13/756,760 Office action dated January 24, 2014 .	☐
	098	U.S. Application No. 13/958,479 Office Action dated March 1, 2016.	☐
	099	U.S. Application No. 14/451,876 Office action dated June 6, 2017.	☐
	100	U.S. Application No. 14/451,876 Office action dated November 23, 2016.	☐
	101	U.S. Application No. 15/623,974 Notice of Allowance dated July 3, 2018.	☐
	102	U.S. Application No. 15/623,974 Office Action dated February 22, 2018.	☐
	103	U.S. Application No. 15/677,772 Final Office Action Mailed June 7, 2018.	☐
	104	U.S. Application No. 15/677,772 Non-Final Office Action Mailed February 8, 2018.	☐
	105	U.S. Application No. 15/892,245 First Action Interview - Office Action mailed July 19, 2018.	☐

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

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 27 of 27

Complete if Known	
Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author(in CAPITAL LETTERS),title of the article(when appropriate), title of the item (book,magazine,journal,serial,symposium,catalog,etc.),date,page(s),volume-issue number(s),publisher, city and/or country where published.	T[2]
	129	ZHANG, et al. Novel Multiplex PCR Assay for Characterization and Concomitant Subtyping of Staphylococcal Cassette Chromosome mec Types I to V in Methicillin-Resistant Staphylococcus aureus. J Clin Microbiol. 2005 Oct;43(10):5026-33.	☐
	130	ZHAO et al. Comprehensive Algorithm for Quantitative Real-Time Polymerase Chain Reaction. Journal of Computational Biology 12(8):1047-1064 (2005).	☐

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /RSN/

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

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/RSN/	2	20100317005	A1	2010-12-16	Hardin et al.	
/RSN/	3	20040248082	A1	2004-12-09	Scallon	
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	Application Number	
	Filing Date	2023-07-13
	First Named Inventor	Emil P. KARTALOV
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	Examiner Name	Not yet assigned
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/RSN/	1	Non-Final Office Action dated November 28, 2022, issued in related U.S. Application No. 16/937,464 (14 pages).	☐
/RSN/	2	Non-Final Office Action dated December 28, 2022, issued in related U.S. Application No. 16/864,744 (15 pages).	☐

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PE2E SEARCH - Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp
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L2	0	"9376464".ap.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2023/12/30 02:26 PM
L3	16	"937464".ap.	(US-PGPUB; USPAT; USOCR)	OR	ON	ON	2023/12/30 02:26 PM
L4	164	("10066263" OR "10068051" OR "10301668" OR "10770170" OR "20020022273" OR "20020146734" OR "20030134320" OR "20030148280" OR "20030148544" OR "20040053230" OR "20040191794" OR "20040248082" OR "20050053950" OR "20050064435" OR "20050106607" OR "20050164264" OR "20050214753" OR "20050250146" OR "20050260640" OR "20060039918" OR "20060216708" OR "20070072211" OR "20070178485" OR "20070231824" OR "20080050737" OR "20080069733" OR "20080096767" OR "20080124705" OR "20090042735" OR "20090048785" OR "20090062129" OR "20100015607" OR "20100041092" OR "20100047924" OR "20100112710" OR "20100120043" OR "20100151443" OR "20100159447" OR "20100210472" OR "20100233686" OR "20100248257" OR "20100261026" OR "20100267064" OR "20100273173" OR	(US-PGPUB; USPAT)	OR	ON	ON	2023/12/30 02:40 PM

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California Institute of Technology - Ex. 2026, Page 1363

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L5	147	kartalov.in. NEAR emil.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)	OR	ON	ON	2023/12/30 02:42 PM
L6	299	rajagopal.in. NEAR aditya.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)	OR	ON	ON	2023/12/30 02:42 PM
L7	1102	scherer.in. NEAR axel.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT)	OR	ON	ON	2023/12/30 02:42 PM
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L19	66	L17 AND L18	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY,	OR	ON	ON	2023/12/30 02:44 PM

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Application	Document	Mailroom Date	Attorney Docket No.
18352112	CTNF	01/05/2024	88DG-373535-US
18352112	1449	01/05/2024	88DG-373535-US
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Application	Document	Mailroom Date	Attorney Docket No.
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/352,112	07/13/2023	Emil P. KARTALOV	88DG-373535-US	3145

30764 7590 10/11/2023

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The time period for reply, if any, is set in the attached communication.

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DOCKETING@SHEPPARDMULLIN.COM
SheppardMullin_Pair@firsttofile.com

IPR2025-01546
California Institute of Technology - Ex. 2026, Page 1371

Decision Granting Request for Prioritized Examination (Track I)	**Application No.** 18/352,112	**Applicant(s)** KARTALOV et al.	
	Examiner KIMBERLY A INABINET	**Art Unit** OPET	**AIA (FITF) Status** No

1. THE REQUEST FILED 13 July 2023 IS **GRANTED** .

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

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

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

A. filing a **petition for extension of time** to extend the time period for filing a reply;

B. filing an **amendment to amend the application to contain more than four independent claims, more than thirty total claims** , or a multiple dependent claim;

C. filing a **request for continued examination** ;

D. filing a notice of appeal;

E. filing a request for suspension of action;

F. mailing of a notice of allowance;

G. mailing of a final Office action;

H. completion of examination as defined in 37 CFR 41.102; or

I. abandonment of the application.

Telephone inquiries with regard to this decision should be directed to KIMBERLY INABINET at (571) 272-4618. In his/her absence, calls may be directed to Petition Help Desk at (571) 272-3282.

/KIMBERLY A INABINET/ Paralegal Specialist, OPET	

IPR2025-01546
California Institute of Technology - Ex. 2026, Page 1372

UNITED STATES PATENT AND TRADEMARK OFFICE

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

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
18/352,112	07/13/2023		1064	88DG-373535-US	30	3

CONFIRMATION NO. 3145

30764
SHEPPARD, MULLIN, RICHTER & HAMPTON LLP
650 Town Center Drive, 10th Floor
Costa Mesa, CA 92626

FILING RECEIPT

OC000000062622256

Date Mailed: 10/03/2023

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

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

Inventor(s)

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

Applicant(s)

CALIFORNIA INSTITUTE OF TECHNOLOGY, Pasadena, CA;

Power of Attorney: The patent practitioners associated with Customer Number 30764

Domestic Priority data as claimed by applicant

This application is a CON of 16/937,464 07/23/2020
which is a CON of 15/914,356 03/07/2018 PAT 10,770,170
which is a CON of 14/451,876 08/05/2014 PAT 10,068,051
which is a CON of 13/756,760 02/01/2013 PAT 8,838,394
which claims benefit of 61/703,093 09/19/2012
and claims benefit of 61/594,480 02/03/2012

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

Permission to Access Application via Priority Document Exchange: Yes

IPR2025-01546
California Institute of Technology - Ex. 2026, Page 1373

Permission to Access Search Results: Yes

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

If Required, Foreign Filing License Granted: 09/28/2023
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 18/352,112**
Projected Publication Date: 01/11/2024
Non-Publication Request: No
Early Publication Request: No
**** SMALL ENTITY ****
Title

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Preliminary Class

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

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

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Title 37, Code of Federal Regulations, 5.11 & 5.15

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IPR2025-01546
California Institute of Technology - Ex. 2026, Page 1376

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 18/352,112

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	64		N/A	
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	280		N/A	
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	320		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	30 minus 20 =	* 10	x 40 =	400	OR		
INDEPENDENT CLAIMS (37 CFR 1.16(h))	3 minus 3 =	*	x =	0			
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			0			
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				0			
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	1064		TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)		RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

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Kathi Vidal

Kathi Vidal
Under Secretary of Commerce for Intellectual Property and
Director of the United States Patent and Trademark Office

Derrick Brent
Deputy Under Secretary of Commerce for Intellectual Property and
Deputy Director of the United States Patent and Trademark Office

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FROM: noreply@uspto.gov
CC: patentcenter_eofficeaction@uspto.gov
SUBJECT: USPTO: Patent Electronic System - Correspondence Notification for Customer Number 30764

Tue Oct 03 05:26:08 EDT 2023

Dear Patent Center Customer:

Correspondence Address:

SHEPPARD, MULLIN, RICHTER &HAMPTON LLP
650 Town Center Drive, 10th Floor
Costa Mesa,CALIFORNIA,92626
UNITED STATES

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The list of documents shown below are provided as a courtesy and is not part of the official file wrapper. The content of the images shown in the Image File Wrapper is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
18352112	APP.FILE.REC	10/03/2023	88DG-373535-US
18352112	WELCOME.LET	10/03/2023	88DG-373535-US

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Sincerely,

The Patent Center Team

PATENT
Attorney Docket No.: 88DG-373535-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor: Emil P. KARTALOV *et al.*	Group Art Unit: Not yet assigned
Serial No.: TBD	Examiner: Not yet assigned
Filed: Herewith	
Title: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

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

NOTICE UNDER 37 C.F.R §1.98(D) REGARDING PREVIOUS SUBMISSION OF FOREIGN PATENTS AND NPL REFERENCES IN AN EARLIER APPLICATION

Commissioner:

Applicant is filing the present paper pursuant to 37 C.F.R §1.98(d), which states as follows:

> (d) A copy of any patent, publication, pending application or other information, as specified in paragraph (a) of this section, listed in an information disclosure statement is required to be provided, even if the patent, publication, pending application or other information was previously submitted to, or cited by, the Office in an earlier application, unless:
>
> > (1) The earlier application is properly identified in the information disclosure statement and is relied on for an earlier effective filing date under 35 U.S.C. 120; and
> >
> > (2) The information disclosure statement submitted in the earlier application complies with paragraphs (a) through (c) of this section.

Pursuant to the above, Applicant hereby notifies the Office of the following:

1. The subject application relies on U.S. Patent Application Serial Nos. 16/937,464, 15/914,356, 14/451,876 and 13/756,760 (the "identified earlier applications") for an earlier effective filing date under 35 U.S.C. §120; and

2. Copies of the foreign patent and non-patent literature references listed on the Information Disclosure Statement submitted herewith were previously provided in the identified earlier applications, and are available in the image file wrapper therefor.

Applicant respectfully submits that the requirements of 37 C.F.R §1.98(d) have been met, and thus additional copies of the foreign patent and non-patent literature references that were previously provided in the identified earlier applications have not been submitted in the subject application.

Respectfully submitted,

Date: July 13, 2023

By: /Jesse A. Salen/
Jesse A. Salen, Reg. No. 72209
Attorney for Applicant

Customer Number 30764
Sheppard, Mullin, Richter & Hampton LLP

IPR2025-01546
California Institute of Technology - Ex. 2026, Page 1381

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PATENT AND TRADEMARK OFFICE

P.O. Box 1450
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ELECTRONIC PAYMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/352,112	**07/13/2023 06:22:52 PM ET**	**88DG-373535-US**

Title of Invention

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	3145	FILED BY	Annie Wong
PATENT CENTER #	62439343	AUTHORIZED BY	Jesse Salen
CUSTOMER #	30764	FILING DATE	-
CORRESPONDENCE ADDRESS	-	FIRST NAMED INVENTOR	Emil P. KARTALOV

Payment Information

PAYMENT METHOD	PAYMENT TRANSACTION ID	PAYMENT AUTHORIZED BY
DA / 504561	E20237CI23437600	Annie Wong

PRE-AUTHORIZED ACCOUNT	PRE-AUTHORIZED CATEGORY
504561	37 CFR 1.17 (Patent application and reexamination processing fees); 37 CFR 1.21 (Miscellaneous fees and charges)

FEE CODE	DESCRIPTION	ITEM PRICE($)	QUANTITY	ITEM TOTAL($)
2830	PROCESSING FEE, EXCEPT IN PROVISIONAL APPLICATIONS	56.00	1	56.00
2817	REQUEST FOR PRIORITIZED EXAMINATION	1680.00	1	1680.00
2111	UTILITY PATENT APPL. SEARCH FEE	280.00	1	280.00
4011	BASIC FILING FEE- UTILITY	64.00	1	64.00
2311	EXAMINATION OF ORIGINAL	320.00	1	320.00

PATENT APPLICATION

2202	EACH CLAIM IN EXCESS OF 20	40.00	10	400.00
			TOTAL AMOUNT:	**$2,800.00**

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

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

PTO/SB/08a (01-10)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(**Not for submission under 37 CFR 1.99)**

Application Number	
Filing Date	2023-07-13
First Named Inventor	Emil P. KARTALOV
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	20060039918	A1	2006-02-23	Albani et al.	
	2	20100317005	A1	2010-12-16	Hardin et al.	
	3	20040248082	A1	2004-12-09	Scallon	
	4	20110183884	A1	2011-07-28	Miller et al.	

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

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NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1	Non-Final Office Action dated November 28, 2022, issued in related U.S. Application No. 16/937,464 (14 pages).	☐
	2	Non-Final Office Action dated December 28, 2022, issued in related U.S. Application No. 16/864,744 (15 pages).	☐

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EXAMINER SIGNATURE

Examiner Signature		Date Considered	

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

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

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

CERTIFICATION STATEMENT

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

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

OR

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

☐ See attached certification statement.

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

☒ A certification statement is not submitted herewith.

SIGNATURE

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

Signature	/Jesse A. Salen/	Date (YYYY-MM-DD)	2023-07-13
Name/Print	Jesse A. Salen	Registration Number	72209

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

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

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

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

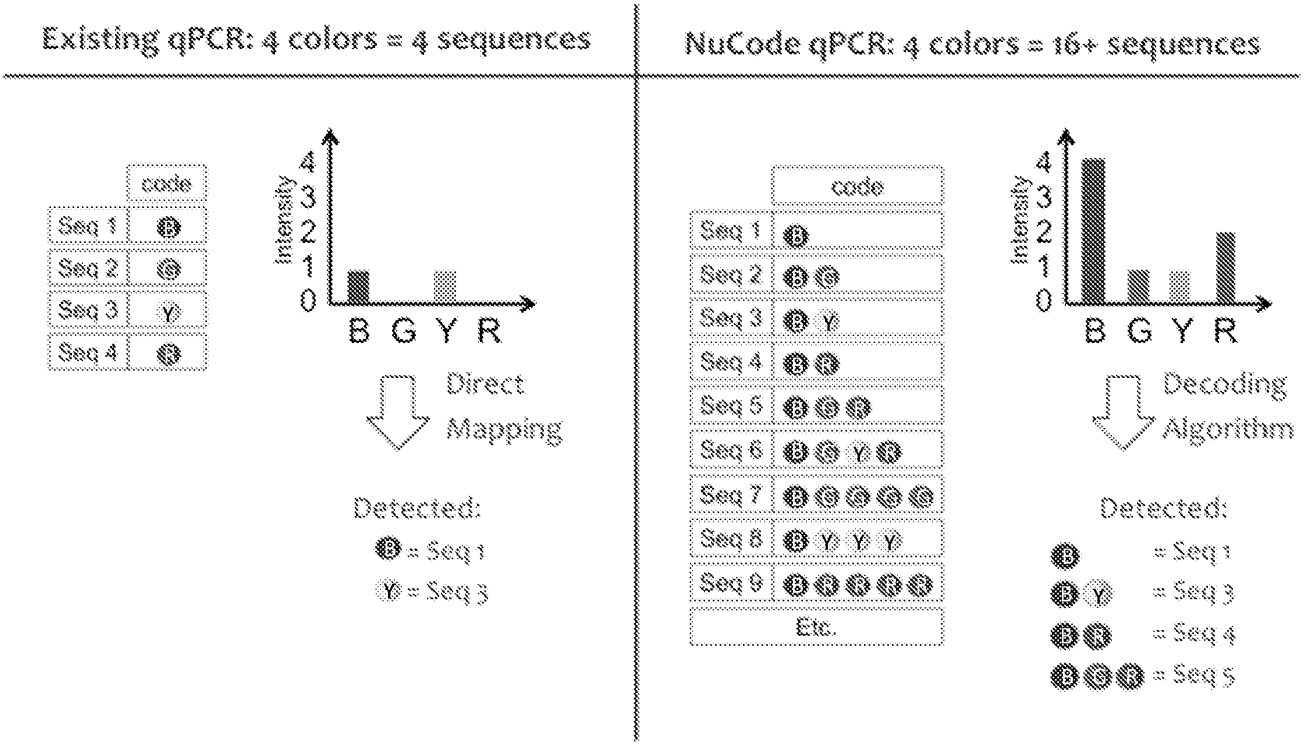
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under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

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

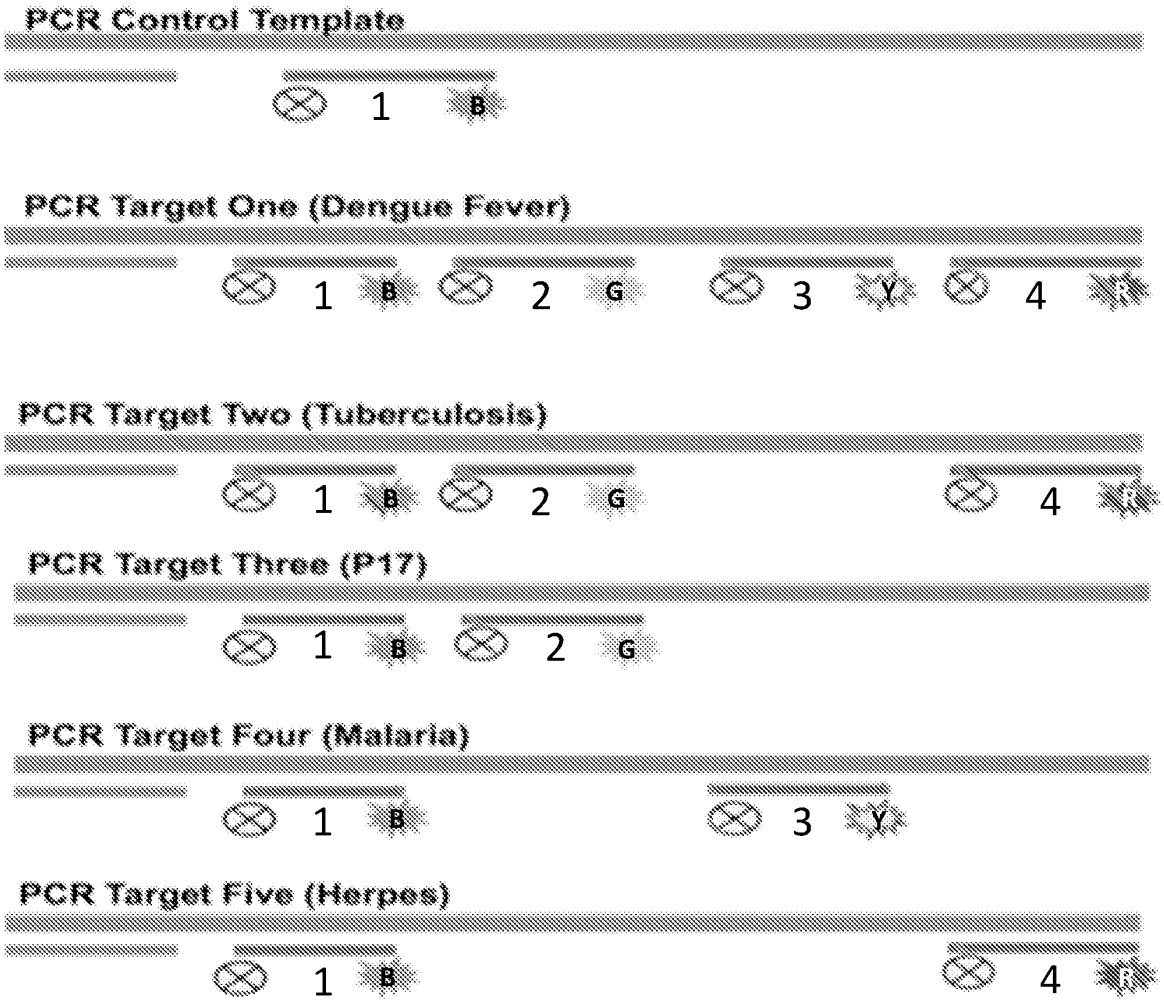
IPR2025-01546
California Institute of Technology - Ex. 2026, Page 1387

Fig. 1



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

Fig. 2



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

Fig. 3

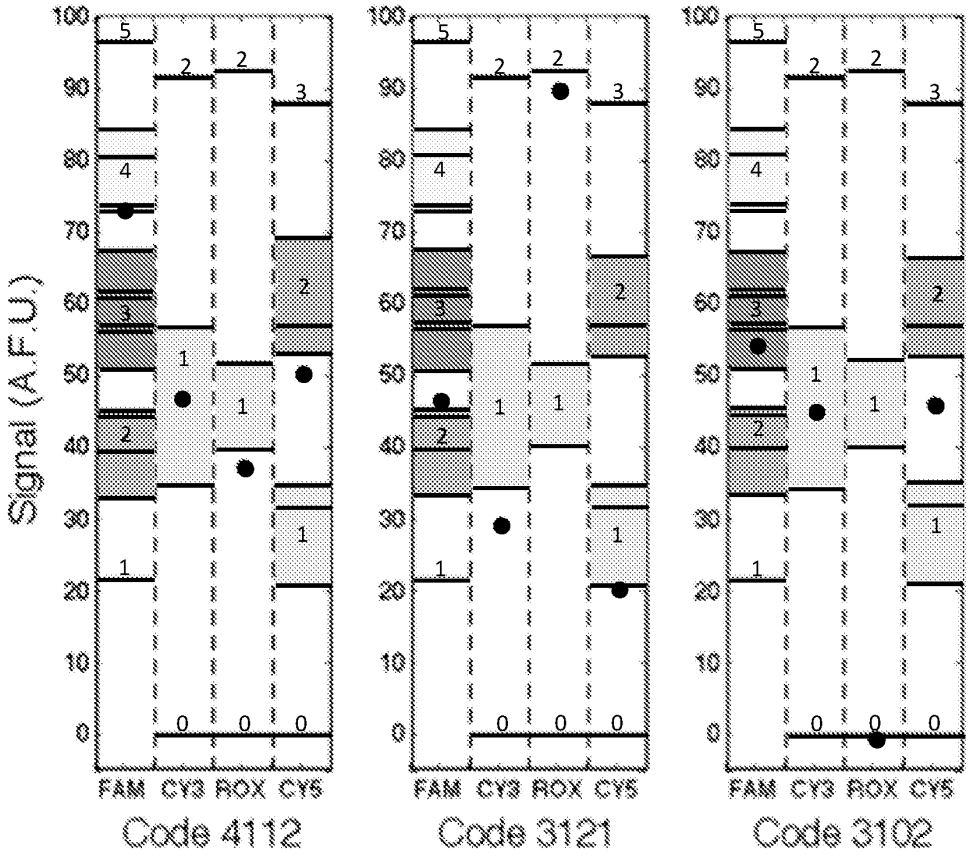


Fig. 4

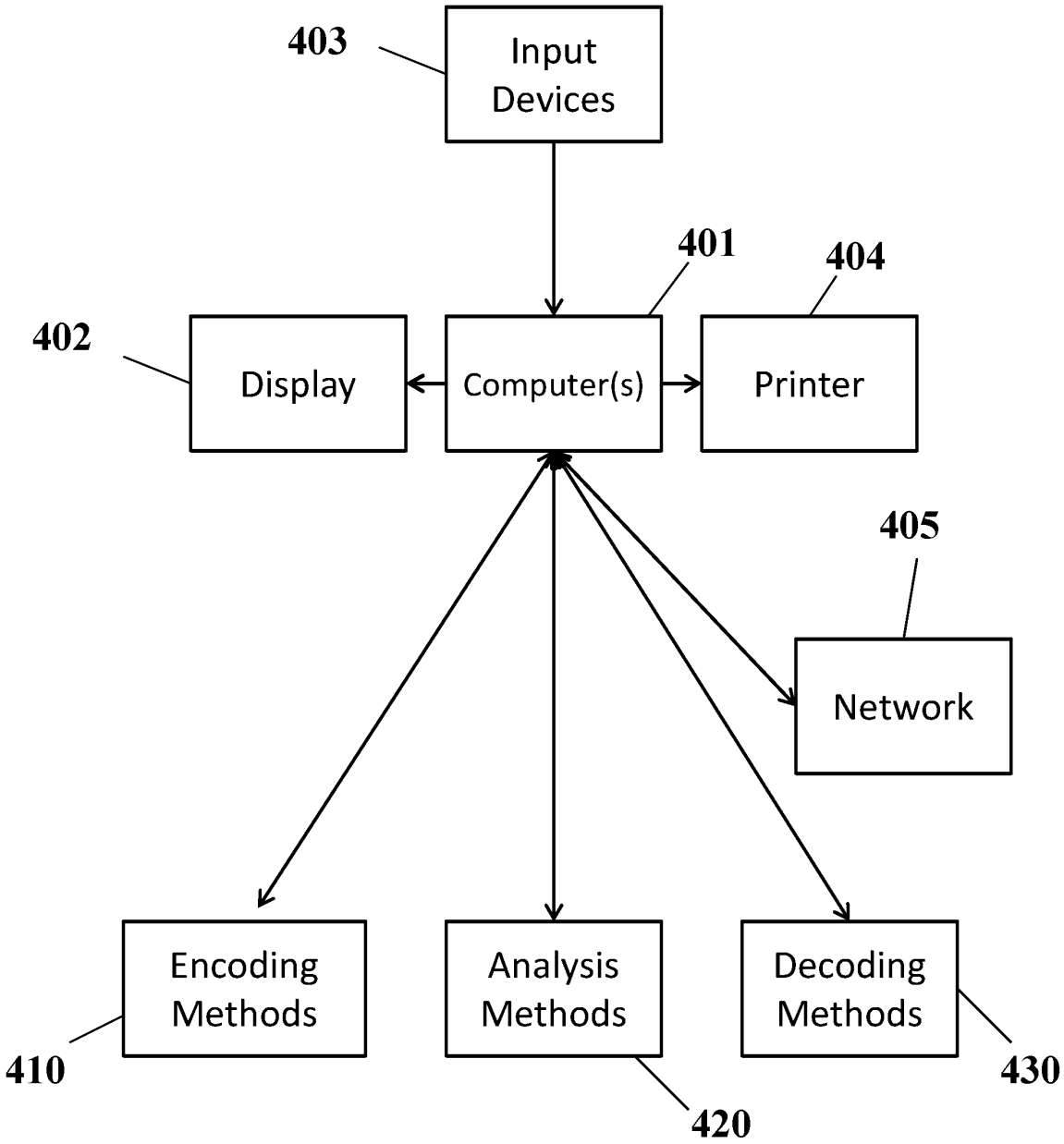

403
Input Devices
401
404
402
Display
Computer(s)
Printer
405
Network
Encoding Methods
Analysis Methods
Decoding Methods
410
420
430

Fig. 5

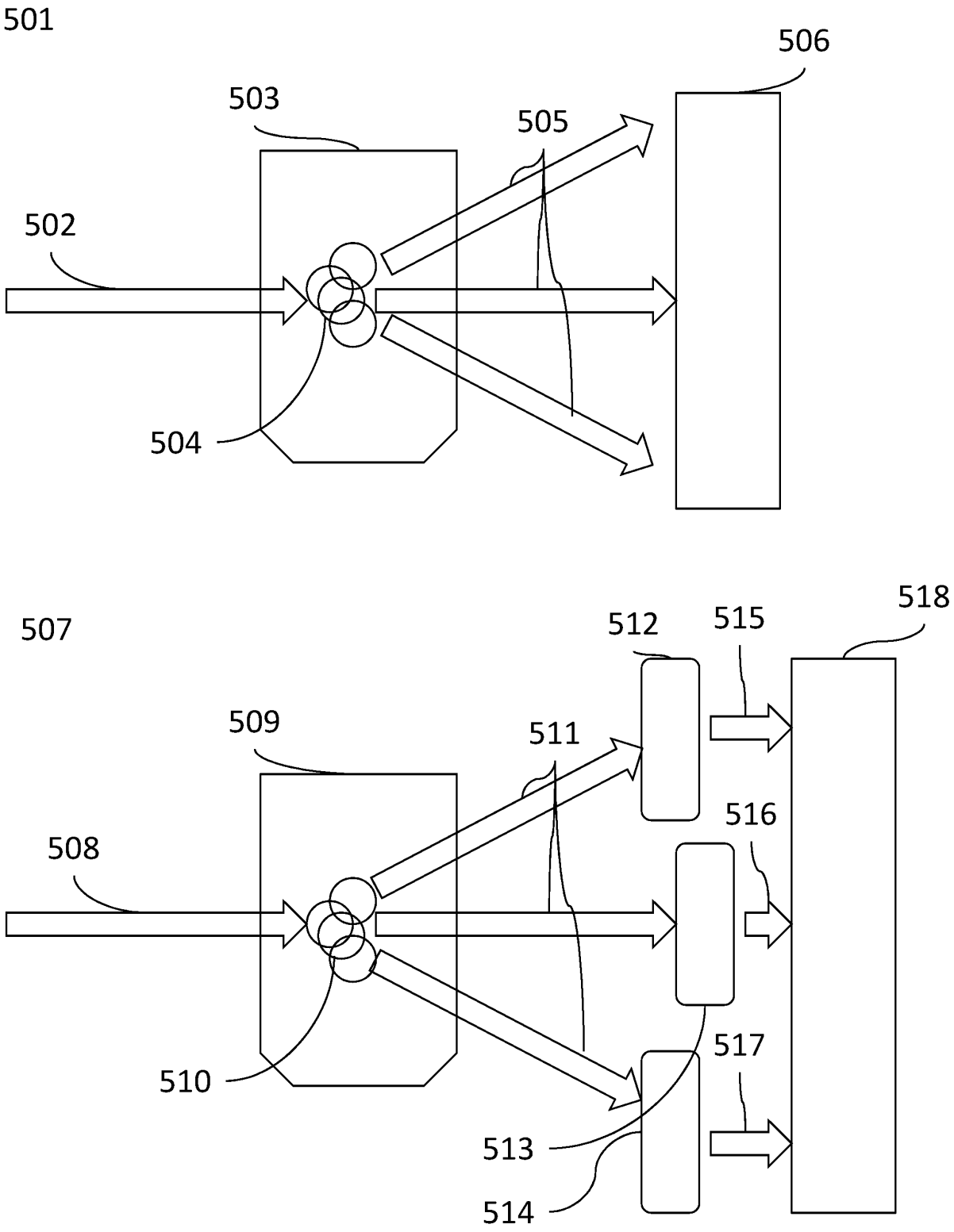

501
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Fig. 6

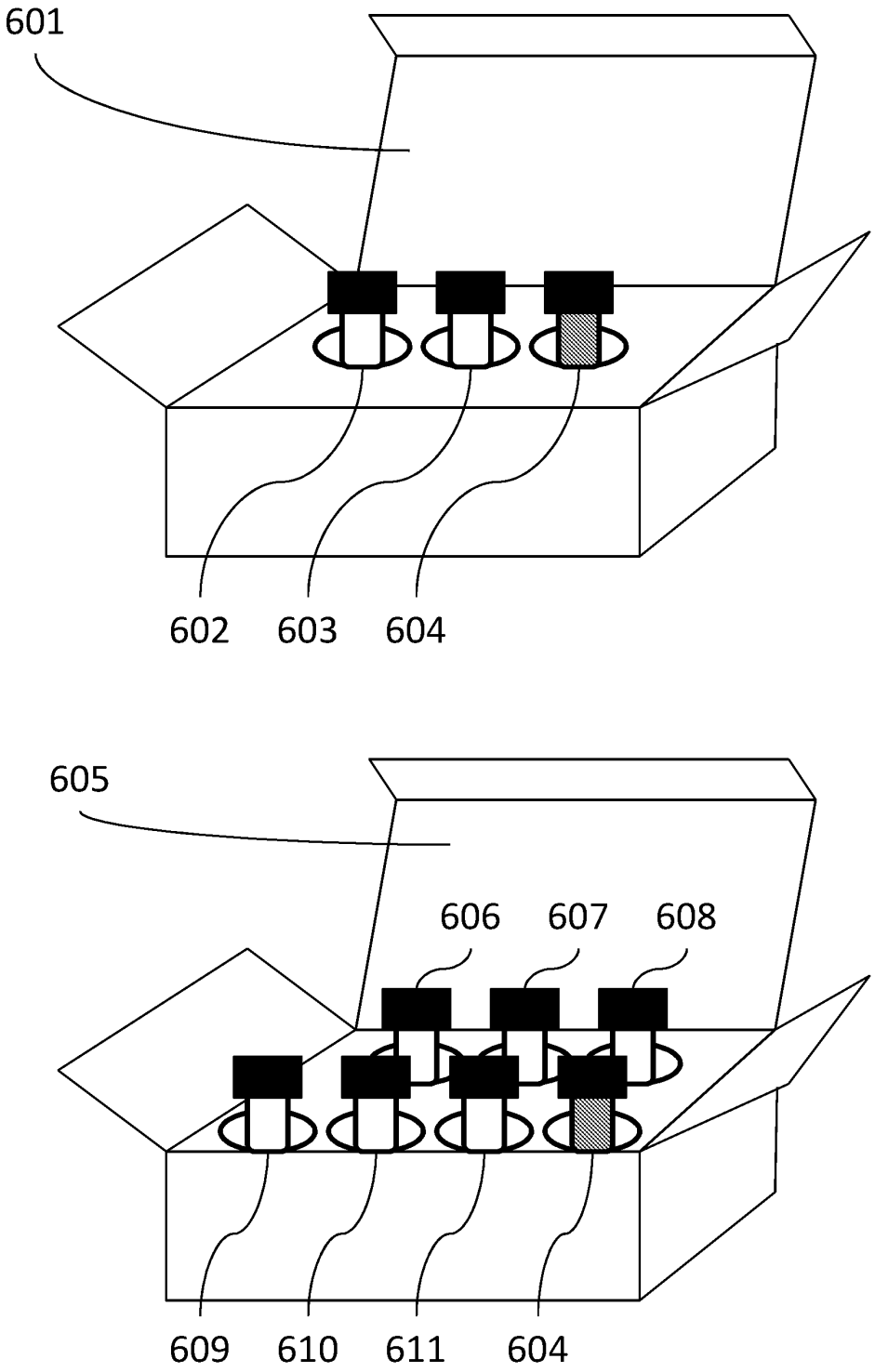


Fig. 7

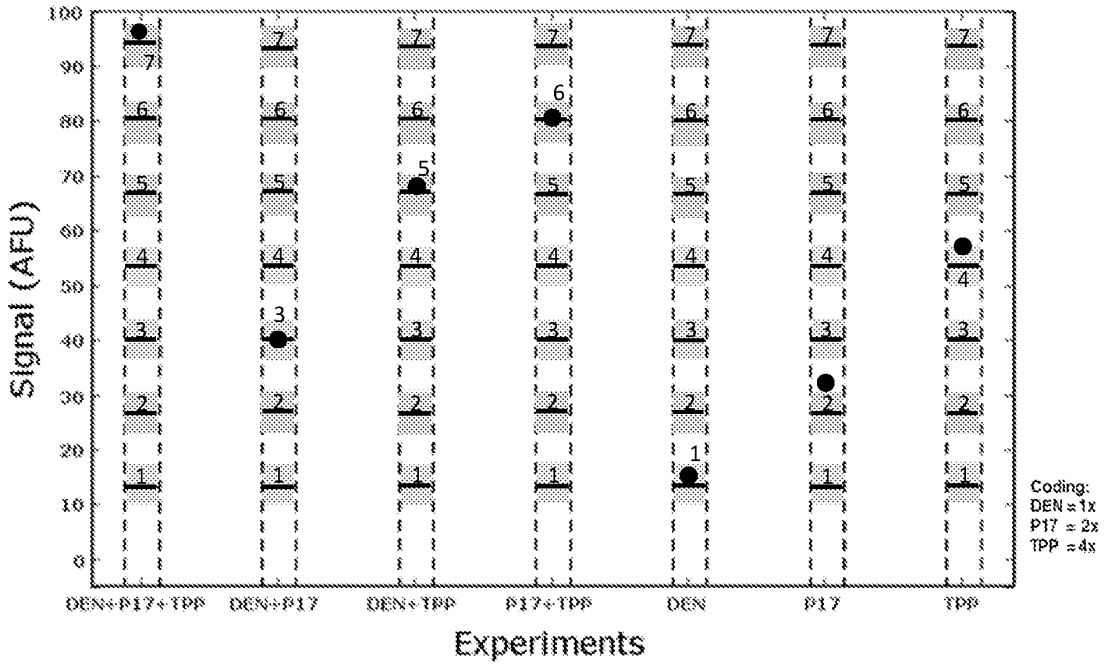
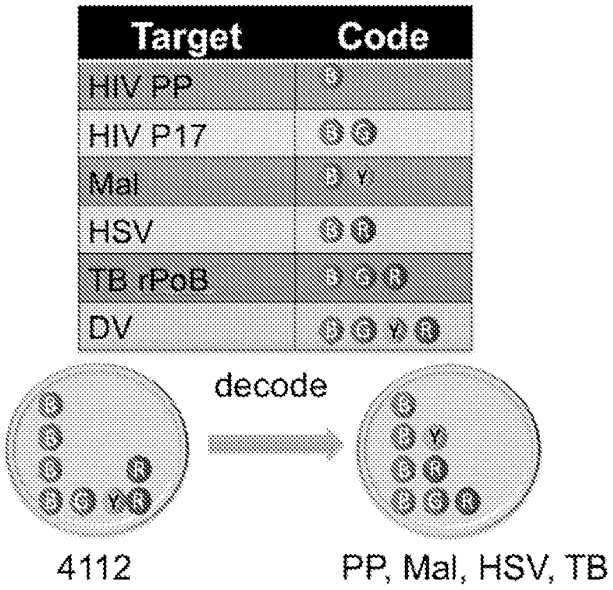
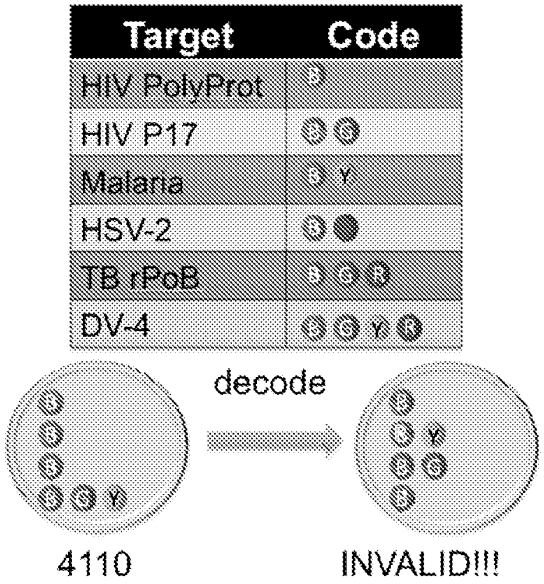

Signal (AFU)
Experiments
DEN+P17+TPP
DEN+P17
DEN+TPP
P17+TPP
DEN
P17
TPP
Coding:
DEN = 1x
P17 = 2x
TPP = 4x

Fig. 8


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


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

UNITED STATES
PATENT AND TRADEMARK OFFICE

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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/352,112	**07/13/2023 06:22:52 PM ET**	**88DG-373535-US**

Title of Invention

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	3145	FILED BY	Annie Wong
PATENT CENTER #	62439343	FILING DATE	-
CUSTOMER #	30764	FIRST NAMED INVENTOR	Emil P. KARTALOV
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Jesse Salen

Documents

TOTAL DOCUMENTS: 15

DOCUMENT		PAGES	DESCRIPTION	SIZE (KB)
ADS_88DG-373535-US.pdf		9	Application Data Sheet	1781 KB
Warning: The attached version of the AIA/14 form is not the current version and data may not fully load to USPTO systems.				
Track_One_88DG-373535-US.pdf		1	Track One Request	116 KB
Application_88DG-373535-US.pdf		99	-	954 KB
Application_88DG-373535-US-SPEC.pdf	(1-92)	92	Specification	926 KB

Application_88DG-373535-US-CLM.pdf	(93-98)	6	Claims	52 KB
Application_88DG-373535-US-ABST.pdf	(99-99)	1	Abstract	20 KB
Drawings_88DG-373535-US.pdf		8	Drawings-only black and white line drawings	487 KB
Declaration_88DG-373535-US.pdf		3	Oath or Declaration filed	209 KB
IDS-1_Transmittal_88DG-373535-US.pdf		2	Transmittal Letter	103 KB
IDS-1_88DG-373535-US.pdf		28	Information Disclosure Statement (IDS) Form (SB08)	18271 KB

Warning: This is not a USPTO supplied IDS fillable form. Data in the form cannot be automatically loaded to other USPTO systems.

IDS-2_88DG-373535-US.pdf		4	Information Disclosure Statement (IDS) Form (SB08)	515 KB
NPL_NFOA_16864744_28Dec2022.pdf		15	Non Patent Literature	516 KB
NPL_NFOA_16937464_28Nov2022.pdf		14	Non Patent Literature	496 KB
SL_Statement_88DG-373535-US.pdf		1	CRF Statement that Sequence Listing text file (CRF) and paper/pdf Sequence Listing are the same	112 KB
SL_Certification_88DG-373535-US.pdf		1	CRF Statement that Sequence Listing text file (CRF) and paper/pdf Sequence Listing are the same	20 KB

373535.xml.xml	-	Sequence Listing (ST.26 XML File)	56 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
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This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

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

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

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C.

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

New International Application Filed with the USPTO as a Receiving Office

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

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/937,464	07/23/2020	Emil P. Kartalov	30KJ-330783-US5	7101

30764 7590 11/28/2022

SHEPPARD, MULLIN, RICHTER & HAMPTON LLP
650 Town Center Drive, 10th Floor
Costa Mesa, CA 92626

EXAMINER	
NEGIN, RUSSELL SCOTT	

ART UNIT	PAPER NUMBER
1672	

NOTIFICATION DATE	DELIVERY MODE
11/28/2022	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

DOCKETING@SHEPPARDMULLIN.COM
SheppardMullin_Pair@firsttofile.com

IPR2025-01546
California Institute of Technology - Ex. 2026, Page 1401

Office Action Summary	Application No. 16/937,464	Applicant(s) Kartalov et al.	
	Examiner RUSSELL S NEGIN	Art Unit 1672	AIA (FITF) Status No

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

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

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

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 22-31 is/are pending in the application.

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

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 22-31 is/are rejected.

8) ☑ Claim(s) 22 is/are objected to.

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

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

Application Papers

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

11)☑ The drawing(s) filed on 23 July 2020 is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

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

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Comments

The present application is being examined under the pre-AIA first to invent provisions. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

Claims 22-31 are pending and examined in the instant Office action.

Information Disclosure Statements

The IDS that has been filed has been considered.

Claim Objections

Claim 22 is objected to because of the following informalities:

Line 15 of claim 22 recites "plurality analytes" which should recite "plurality **of** analytes".

Appropriate correction is required.

Claim Rejections - 35 USC § 112(b) - Indefiniteness

The following is a quotation of 35 U.S.C. 112(b):

(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 22-31 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor (or for applications subject to pre-AIA 35 U.S.C. 112, the applicant), regards as the invention.

Claim 22 recites that a plurality of analytes corresponds with a plurality of respiratory viral pathogens. It is unclear as to the metes and bounds of this correspondence. For example, it is unclear whether one analytes corresponds with one respiratory viral pathogen or two analytes corresponds with one respiratory pathogen. For the purpose of examination, it is interpreted that any ratio of correspondence of analytes to pathogens meets this limitation of the claims.

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was

not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

35 U.S.C. 103 Rejection #1:

Claims 22-24 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Han et al. [Nature Biotechnology, volume 19, 2001, pages 631-635; on IDS] in view of Scallon [US PGPUB 2004/0248082 A1].

Claim 22 is drawn to a method of unambiguously detecting any unique combination of presence or absence of a plurality of analytes corresponding with a plurality of respiratory viral pathogens. The method comprises providing a sample from a subject comprising, or potentially comprising, the plurality of analytes. The method comprises forming a mixture of the sample and hybridization probes. At least one of the hybridization probes is configured to bind to at least one of the plurality of analytes corresponding with a respiratory viral pathogen. The hybridization probes comprise at least one fluorophore and at most four fluorophores. The method comprises exciting the at least one fluorophore to generate one or more signals if one or more of the plurality of analytes is present. The one or more signals comprise at least one signal generated by excitement of the one or more fluorophores. The method comprises measuring the one or more signals to generate a cumulative intensity measurement. The cumulative intensity measurement corresponds to the presence of a unique combination of presence or absence of the plurality of analytes in the sample. The method comprises determining whether the at least on of the plurality of analytes

corresponds with the respiratory viral pathogen is present (in any unique combination of presence or absence) based on the cumulative intensity measurement. The method does not require any step of immobilization of the plurality of polynucleotide analytes or mass spectrometry.

Claim 23 is further limiting wherein the respiratory viral pathogen is selected from influenza virus or RSV.

Claim 24 is further limiting wherein the plurality of analytes are derived from at least three respiratory viral pathogens.

The document of Han et al. studies quantum dot tagged microbeads for multiplexed optical coding of biomolecules [title]. Figure 5 on page 634 of Han et al. illustrates three different polynucleotide analytes labeled with three different combinations of three different fluorophores. The bottom three polynucleotide analytes are hybridized to complementary polynucleotide probes. When the sample undergo spectroscopy, the optical code on the right panel of the figure is the cumulative measurement of the presence/absence combination of fluorophores and each of the three polynucleotide analytes. The technique of Han et al. does not involve immobilization or mass spectrometry.

Han et al. does not teach identifying respiratory viral pathogens.

The document of Scallon studies analytes that encode RSV [abstract]. The figures of Scallon illustrates a subset of these analytes. Paragraph 177 of Scallon teaches at least three respiratory viral pathogens comprising influenza virus.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the assay of Han et al. by use of analytes corresponding to the respiratory viral pathogens of Scallon wherein the motivation would have been that the respiratory viral pathogens give a physiological application to the empirical assay of Han et al. [abstract and paragraph 177 of Scallon].

35 U.S.C. 103 Rejection #2:

Claims 25-26 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Han et al. in view of Scallon as applied to claims 22-24 above, in further view of Craig et al. [Nucleic Acids Research, volume 18, 1990, pages 2653-2660; on IDS].

Claims 25 and 26 are further limiting wherein the analytes are comprise at least five and seven analytes, respectively.

Han et al. Scallon make obvious using combinatorial hybridization and fluorophore labeling to identify presence/absence patterns of polynucleotide analytes from respiratory viral pathogens, as discussed above.

Han et al. and Scallon do not teach at least five or seven analytes.

The document of Craig et al. studies ordering of cosmid clones in HSV-I genome using fingerprinting by hybridization [title]. Figure 1 of Craig et al. lists the 22 analytes used to identify the isomer of HSV-1 genome.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the labeling to identify respiratory viral pathogens of Han et al. and Scallon by use of the 22 analytes of Craig et al. wherein the motivation would

have been that 22 analytes gives more complete coverage of the HSV-I genome [Figure 5 of Craig et al.]. There would have been a reasonable expectation of success in combining Scallon and Craig et al. because both studies are analogously applicable to identifying viruses using analytes.

35 U.S.C. 103 Rejection #3:

Claims 27-31 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Han et al. in view of Scallon as applied to claims 22-24 above, in further view of Miller et al. [US PGPUB 2011/0183884 A1].

Claims 27-31 recite taking a bodily fluid as sample, determining through the presence of analyte the presence of a respiratory viral pathogen, and making a clinical decision to treat the patient with an anti-viral drug if the pathogen is present.

Han et al. Scallon make obvious using combinatorial hybridization and fluorophore labeling to identify presence/absence patterns of polynucleotide analytes from respiratory viral pathogens, as discussed above.

Han et al. and Scallon do not teach diagnosis and treatment of the disease caused by the pathogen.

The document of Miller et al. studies antiviral peptides for kinase inhibition [title]. Paragraphs 39-40 in Example 3 of Miller et al. teach diagnosing the presence of influenza in a blood sample and treated the patient with an antiviral drug.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the labeling to identify respiratory viral pathogens of Han et al. and Scallon by use of the diagnosis and treatment of canine influenza of Miller et

al. wherein the motivation would have been that anti-viral drugs solve a health problem caused by the presence of respiratory viral pathogens [Example 3 of Miller et al.].

Double Patenting

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

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) -

706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

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

Double Patenting Rejection #1:

Claims 22 and 29 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1 and 6, respectively, of U.S. Patent No. 10,068,051 B2 [on IDS] in view of Scallon. Although the claims at issue are not identical, they are not patentably distinct from each other because both sets of claims are directed to combinatorial manner of identifying the contents of mixtures of polynucleotide analytes without immobilization or mass spectrometry.

The claims of ‘051 do not teach applying the analysis to respiratory viral pathogens.

The document of Scallon studies analytes that encode RSV [abstract]. The figures of Scallon illustrates a subset of these analytes. Paragraph 177 of Scallon teaches at least three respiratory viral pathogens comprising influenza virus.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the assay of the claims of '051 by use of analytes corresponding to the respiratory viral pathogens of Scallon wherein the motivation would have been that the respiratory viral pathogens give a physiological application to the empirical assay of Han et al. [abstract and paragraph 177 of Scallon].

Double Patenting Rejection #2:

Claim 22 is rejected on the ground of nonstatutory double patenting as being unpatentable over claim 1, of U.S. Patent No. 10,770,170 B2 [on IDS] in view of Scallon. Although the claims at issue are not identical, they are not patentably distinct from each other because both sets of claims are directed to combinatorial manner of identifying the contents of mixtures of polynucleotide analytes without immobilization or mass spectrometry.

The claim of '170 do not teach applying the analysis to respiratory viral pathogens.

The document of Scallon studies analytes that encode RSV [abstract]. The figures of Scallon illustrates a subset of these analytes. Paragraph 177 of Scallon teaches at least three respiratory viral pathogens comprising influenza virus.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the assay of the claim of '170 by use of analytes

corresponding to the respiratory viral pathogens of Scallon wherein the motivation would have been that the respiratory viral pathogens give a physiological application to the empirical assay of Han et al. [abstract and paragraph 177 of Scallon].

Related Prior Art

The study of Wang et al. [Journal of the American Chemical Society, volume 127, 2005, pages 15664-15665; on IDS] studies attaching molecular beacons to oligonucleotides. However, Wang et al. does not teach the assay recited in the claims.

The document of Tyagi et al. [Nature Biotechnology, volume 18, 2000, pages 1191-1196; on IDS] also studies using molecular beacons to identify analytes. However, Tyagi et al. does not teach the assay recited in the claims.

E-mail Communications Authorization

Per updated USPTO Internet usage policies, Applicant and/or applicant's representative is encouraged to authorize the USPTO examiner to discuss any subject matter concerning the above application via Internet e-mail communications. See MPEP 502.03. To approve such communications, Applicant must provide written authorization for e-mail communication by submitting the following statement via EFS-Web (using PTO/SB/439) or Central Fax (571-273-8300):

> Recognizing that Internet communications are not secure, I hereby authorize the USPTO to communicate with the undersigned and practitioners in accordance with 37 CFR 1.33 and 37 CFR 1.34 concerning any subject matter of this application by video conferencing, instant messaging, or electronic mail. I understand that a copy of these communications will be made of record in the application file.

Written authorizations submitted to the Examiner via e-mail are **NOT** proper. Written authorizations must be submitted **via EFS-Web** (using PTO/SB/439) **or Central Fax**

(571-273-8300). A paper copy of e-mail correspondence will be placed in the patent application when appropriate. E-mails from the USPTO are for the sole use of the intended recipient, and may contain information subject to the confidentiality requirement set forth in 35 USC § 122. See also MPEP 502.03.

Conclusion

No claim is allowed.

Any inquiry concerning this communication or earlier communications from the Examiner should be directed to Russell Negin, whose telephone number is (571) 272-1083. This Examiner can normally be reached from Monday through Thursday from 9:00 am to 5 pm and variable hours on Fridays.

If attempts to reach the Examiner by telephone are unsuccessful, the Examiner's Supervisor, Karlheinz Skowronek, Supervisory Patent Examiner, can be reached at (571) 272-9047.

Information regarding the status of the application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only.

/RUSSELL S NEGIN/
Primary Examiner, Art Unit 1672
20 November 2022

Notice of References Cited	Application/Control No. 16/937,464	Applicant(s)/Patent Under Reexamination Kartalov et al.	
	Examiner RUSSELL S NEGIN	Art Unit 1672	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-20040248082-A1	12-2004	Scallon, Bernard	C07K16/1027	435/69.3
*	B	US-20110183884-A1	07-2011	Miller; Kent D.	A61P35/00	514/1.1
	C					
	D					
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	G					
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	I					
	J					
	K					
	L					
	M					

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

IPR2025-01546
California Institute of Technology - Ex. 2026, Page 1414

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Docket No: 88DG-373535

In re patent application of

CALIFORNIA INSTITUTE OF TECHNOLOGY

Serial No.: UNASSIGNED

Filed: CONCURRENTLY HEREWITH

For: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

STATEMENT TO SUPPORT FILING AND SUBMISSION IN ACCORDANCE WITH 37 C.F.R. §§1.831-1.835

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

To Whom It May Concern:

In connection with the Sequence Listing submitted concurrently herewith, the undersigned hereby states that the submission, in accordance with 37 C.F.R. §1.835(a)(4), does not include new matter.

Respectfully submitted,

July 12, 2023
Date

/tara rix/
Tara Rix

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/864,744	05/01/2020	Emil P. Kartalov	30KJ-330783-US4	9929

30764 7590 12/28/2022

SHEPPARD, MULLIN, RICHTER & HAMPTON LLP
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EXAMINER	
NEGIN, RUSSELL SCOTT	

ART UNIT	PAPER NUMBER
1672	

NOTIFICATION DATE	DELIVERY MODE
12/28/2022	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

DOCKETING@SHEPPARDMULLIN.COM
SheppardMullin_Pair@firsttofile.com

Office Action Summary	Application No. 16/864,744	Applicant(s) Kartalov et al.	
	Examiner RUSSELL S NEGIN	Art Unit 1672	AIA (FITF) Status No

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

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

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

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 21-40 is/are pending in the application.

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

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 21-40 is/are rejected.

8) ☑ Claim(s) 33 and 37 is/are objected to.

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

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

Application Papers

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

11)☑ The drawing(s) filed on 01 May 2020 is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

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

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Comments

The present application is being examined under the pre-AIA first to invent provisions. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

Claims 21-40 are pending and examined in the instant Office action.

Information Disclosure Statement

The IDS filed on 3/25/2021 has been considered.

Claim Objections

Claims 33 and 37 are objected to because of the following informalities:

Claim 33 does not end with a period.

Claim 37 recites “The kit of any of claims 34” which should read “The kit of claim 34”.

Appropriate correction is required.

Claim Rejections - 35 USC § 112(b) - Indefiniteness

The following is a quotation of 35 U.S.C. 112(b):

> (b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.
>
> The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 30 and 32-40 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor (or for applications subject to pre-AIA 35 U.S.C. 112, the applicant), regards as the invention.

Claim 30 recites the limitation "said analyte" in line 2. There is insufficient antecedent basis for this limitation in the claim. While the claim previously recites the plural term of analytes, it is unclear as to which of the plurality of analytes is “said analyte.” For the purpose of examination, any analyte is interpreted to be “said analyte.”

Claim 32 recites the limitation "said coding scheme" in line 1. There is insufficient antecedent basis for this limitation in the claim. The phrase “coding scheme” is not previously precited in the claim. For the purpose of examination, “said coding scheme” is interpreted to correspond to the presence/absence pattern of analytes.

Claim 33 recites the limitation "said coding scheme" in line 1. There is insufficient antecedent basis for this limitation in the claim. The phrase “coding scheme” is not previously precited in the claim. For the purpose of examination, “said coding scheme” is interpreted to correspond to the presence/absence pattern of analytes.

Claim 34 is recites the limitation "the first value" in the final line of the claim. There is insufficient antecedent basis for this limitation in the claim. For the purpose of

examination, “the first value” is interpreted to correspond to a value of one component of a fluorescent signal.

Claim 36 recites the limitation "F" in line 2. There is insufficient antecedent basis for this limitation in the claim. The variable “F” is not previously recited or defined in the claim. For the purpose of examination, it is interpreted that “F” corresponds to a number of wavelength components.

Claim 39 recites the limitation "the number of analytes" in lines 1 and 2. There is insufficient antecedent basis for this limitation in the claim. The phrase “the number of analytes” is not previously recited in the claim. For the purpose of examination, the phrase is interpreted to correspond to M analytes.

Claim 39 recites the limitation "the plurality of analytes" in line 2. There is insufficient antecedent basis for this limitation in the claim. The phrase “the plurality of analytes” is not previously recited in the claim. The claim does not require the variable M in M analytes to be more than one.

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter

of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

35 U.S.C. 103 Rejection #1:

Claims 21-25, 27, 29-30, and 32-40 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Han et al. [Nature Biotechnology, volume 19, 2001, pages 631-835; on IDS] in view of Lin et al. [WO 2012/058628 A2; on IDS].

Claim 21 is drawn to an assay that is capable of unambiguously detecting the presence or absence of each of at least M analytes. The assay requires that each of the M analytes is encoded as a value of one component of a fluorescent signal. The assay requires that the fluorescent signal comprises F wavelength components. The assay is capable of detecting the presence or absence of each of the at least M analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis, based on detection of the F wavelength components. M is greater than F.

Claim 32 is further limiting requiring the coding scheme to be innately non-degenerate or non-degenerate by construction.

Claims 34-36 are drawn to similar subject matter as claim 32, except as a kit.

The document of Han et al. studies quantum-dot tagged microbeads for multiplexed optical coding of biomolecules [title]. Figure 5 of Han et al. illustrates four oligonucleotides analytes (M=4) attached to microbeads with different combinations of 3 types of quantum dots- red, green, and blue (F=3). The type of optical code as a result of spectroscopy indicates the type of analyte being examined. Han et al. does not use immobilization, separation, mass spectrometry, or melting curve analysis.

Han et al. does not teach detecting cumulative non-degenerate combinations of analytes.

The document of Lin et al. studies nucleic acid nanostructure barcode probes [title]. Figure 2 of Lin et al. illustrates the cumulative non-degenerate combination of each of 27 different combinations of the three fluorophores (R, G, and B) within an image. Pages 7 and 8 of Lin et al. describe Figure 2 of Lin et al.

With regard to claims 22-23, Han et al. and Lin et al. teach red, green, and blue components to the signal. Figure 6 of Han et al. illustrates a range of intensities associated with each component.

With regard to claims 24-25 and 37-39, Figure 5 of Han et al. illustrates hybridization probes for different analytes wherein each analyte comprises a different combination of fluorophores. Figure 2 of Lin et al. teaches that the number of hybridization probes is greater than the number of analytes.

Wirth regard to claim 27, Figure 5 of Han et al. illustrates three components of the signals used to detect each analyte.

With regard to claim 29, Figure 5 of Han et al. teaches that each analyte comprises a polynucleotide.

With regard to claim 30, page 31 of Lin et al. teaches the presence of a computer to analyze spectral data.

With regard to claims 32-33, Figure 2 of Lin et al. teaches a histogram of non-degenerate counts of presence the 27 different combinations of fluorophores in an image.

With regard to claim 40, the different sizes of the bars in the histogram in Figure 2 of Lin et al. teaches that the analyte specific reagents are each present at a different amount.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the quantum dot hybridization assay of Han et al. by use of the cumulative non-degenerate fluorophore assay of Lin et al. wherein the motivation would have been that the cumulative and non-degenerate nature of the assay of Lin et al. adds mathematical and empirical tools to the assay of Han et al. [Figure 2 of Han et al.]. There would have been a reasonable expectation of success in combining Han et

al. and Lin et al. because both studies analogously pertain to assays involving colored fluorophores or quantum dots.

35 U.S.C. 103 Rejection #2:

Claims 26 and 31 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Han et al. in view of Lin et al. as applied to claims 21-25, 27, 29-30, and 32-40 above, in further view of Albani et al. [US PGPUB 2006/0039918 A1].

Claim 26 is further limiting wherein the signal is generated during PCR.

Claim 31 is further limiting wherein the assay is performed using a computer and thermal cycler.

Han et al. and Lin et al. make obvious fluorophore and quantum dot assays to detect presence and absence of analytes, as discussed above.

Han et al. and Lin et al. do not teach PCR, computers, and thermal cyclers.

The document of Albani et al. studies stress proteins and peptides [title]. Paragraph 184 of Albani et al. relates fluorophores, PCR, and thermal cyclers.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the quantum dot hybridization assay of Han et al. and the cumulative non-degenerate fluorophore assay of Lin et al. by use of the PCR, computers, and thermal cyclers of Albani et al. wherein the motivation would have been that Albani et al. adds empirical tools that facilitate the analysis of fluorophores [paragraph 184 of Albani et al.].

35 U.S.C. 103 Rejection #3:

Claim 28 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Han et al. in view of Lin et al. as applied to claims 21-25, 27, 29-30, and 32-40 above, in further view of Hardin et al. [US PGPUB 2010/0317005 A1].

Claim 28 is further limiting wherein the fluorescence signals are used to generate chromatograms.

Han et al. and Lin et al. make obvious fluorophore and quantum dot assays to detect presence and absence of analytes, as discussed above.

Han et al. and Lin et al. do not teach using fluorescence signals to generate chromatograms.

The document of Hardin et al. studies modified nucleotides [title]. Paragraph 143 of Hardin et al. relates fluorescence signals to chromatograms.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the quantum dot hybridization assay of Han et al. and the cumulative non-degenerate fluorophore assay of Lin et al. by use of the chromatograms of Hardin et al. wherein the motivation would have been that Hardin et al. adds empirical tools that facilitate the analysis of fluorophores [paragraph 143 of Hardin et al.].

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the “right to exclude” granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at

least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP § 2146 *et seq.* for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

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

more information about eTerminal Disclaimers, refer to

www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

Double Patenting Rejection #1:

Claims 21, 23, 26, 27, and 29-34 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1, 3, 7-8, 11-12, 15-17, and 20, respectively, of U.S. Patent No. 8,838,394. Although the claims at issue are not identical, they are not patentably distinct from each other because both sets of claims are drawn to assays for detection cumulative presence/absence patterns of analytes associated with fluorophores without using immobilization or mass spectrometry. While the claims of '394 recite more limitations than the instantly rejected claims, the claims of '394 anticipate the instantly rejected claims.

Double Patenting Rejection #2:

Claims 21, 26, and 30-31 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1, 4-5, and 9, respectively, of U.S. Patent No. 10,068,051 B2. Although the claims at issue are not identical, they are not patentably distinct from each other because both sets of claims are drawn to assays for detection cumulative presence/absence patterns of analytes associated with fluorophores without using immobilization or mass spectrometry. While the claims of '394 recite more limitations than the instantly rejected claims, the claims of '051 anticipate the instantly rejected claims.

Double Patenting Rejection #3:

Claims 21, 26, and 30 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1, 7, and 13, respectively, of U.S. Patent No. 10,770,170 B2. Although the claims at issue are not identical, they are not patentably distinct from each other because both sets of claims are drawn to assays for detection cumulative presence/absence patterns of analytes associated with fluorophores without using immobilization or mass spectrometry. While the claims of '394 recite more limitations than the instantly rejected claims, the claims of '170 anticipate the instantly rejected claims.

Related Prior Art

The prior art of Geiss et al. [WO 2008/124847; on IDS] teach targeting sequences using coding via labeling. However, Geiss et al. does not teach the technique in the recited claims.

E-mail Communications Authorization

Per updated USPTO Internet usage policies, Applicant and/or applicant's representative is encouraged to authorize the USPTO examiner to discuss any subject matter concerning the above application via Internet e-mail communications. See MPEP 502.03. To approve such communications, Applicant must provide written authorization for e-mail communication by submitting the following statement via EFS-Web (using PTO/SB/439) or Central Fax (571-273-8300):

> Recognizing that Internet communications are not secure, I hereby authorize the USPTO to communicate with the undersigned and practitioners in accordance with 37 CFR 1.33 and 37 CFR 1.34 concerning any subject matter of this application by video conferencing,

> instant messaging, or electronic mail. I understand that a copy of these communications will be made of record in the application file.

Written authorizations submitted to the Examiner via e-mail are **NOT** proper. Written authorizations must be submitted **via EFS-Web** (using PTO/SB/439) **or Central Fax** (571-273-8300). A paper copy of e-mail correspondence will be placed in the patent application when appropriate. E-mails from the USPTO are for the sole use of the intended recipient, and may contain information subject to the confidentiality requirement set forth in 35 USC § 122. See also MPEP 502.03

Conclusion

No claim is allowed.

Any inquiry concerning this communication or earlier communications from the Examiner should be directed to Russell Negin, whose telephone number is (571) 272-1083. This Examiner can normally be reached from Monday through Thursday from 9:00 am to 5 pm and variable hours on Fridays.

If attempts to reach the Examiner by telephone are unsuccessful, the Examiner's Supervisor, Karlheinz Skowronek, Supervisory Patent Examiner, can be reached at (571) 272-9047.

Information regarding the status of the application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only.

/RUSSELL S NEGIN/
Primary Examiner, Art Unit 1672
22 December 2022

Notice of References Cited	Application/Control No. 16/864,744	Applicant(s)/Patent Under Reexamination Kartalov et al.	
	Examiner RUSSELL S NEGIN	Art Unit 1672	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-20060039918-A1	02-2006	Albani; Salvatore	A61P25/00	424/185.1
*	B	US-20100317005-A1	12-2010	Hardin; Susan H.	C12Q1/6869	435/6.14
	C					
	D					
	E					
	F					
	G					
	H					
	I					
	J					
	K					
	L					
	M					

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
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	Q					
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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
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*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

IPR2025-01546
California Institute of Technology - Ex. 2026, Page 1430

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

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Emil P. KARTALOV *et al.*

Application No.: Not Yet Assigned | Confirmation No.: Not Yet Assigned

Filed: Concurrently Herewith | Art Unit: Not Yet Assigned

For: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS | Examiner: Not Yet Assigned

STATEMENT PURSUANT TO 37 C.F.R. § 1.821

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

Dear Commissioner:

The sequence listing submitted herein in connection with the above-referenced patent application is a computer readable form of the Sequence Listing that satisfies the requirements of both 37 C.F.R. § 1.821(c) and § 1.821(e). Therefore, no paper/PDF copy is separately submitted.

Further, Applicant submits that no new matter is added in the sequence listing, which includes the same sequences as the sequence listing filed with the PCT or parent application. Accordingly, entry of the updated sequence listing is respectfully requested.

Dated: July 13, 2023

Respectfully submitted,

By /Jesse A. Salen/
Jesse A. Salen
Registration No.: 72209

SHEPPARD, MULLIN, RICHTER & HAMPTON LLP
12275 El Camino Real, Suite 100
San Diego, CA 92130
(858) 720-8900
Attorney For Applicant

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 27

Complete if Known	
Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

U. S. PATENT DOCUMENTS

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

Examiner Signature		Date Considered	

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Sheet 2 of 27

Complete if Known	
Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

U. S. PATENT DOCUMENTS

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	022	US-6534274	03-18-2003	BECKER; Michael M. et al.	
	023	US-6548259	04-15-2003	WARD; David C. et al.	
	024	US-6642062	11-04-2003	KAUVAR; Lawrence M. et al.	
	025	US-6893875	05-17-2005	TSUJI; Yasuhiro et al.	
	026	US-7101663	09-05-2006	GODFREY; Tony E. et al.	
	027	US-7141377	11-28-2006	GELFAND; David H. et al.	
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	030	US-7410764	08-12-2008	GOCKE; Christopher et al.	
	031	US-7413708	08-19-2008	MAYRAND; Paul E.	
	032	US-7473767	01-06-2009	DIMITROV; Krassen	
	033	US-7507575	03-24-2009	BEDINGHAM; William [Us] et al.	
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	035	US-7667024	02-23-2010	MAO; FEI et al.	
	036	US-7671184	03-02-2010	HAENER; Robert et al.	
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Sheet 3 of 27

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First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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	042	US-7930106	04-19-2011	CARRICK; James M. [Us] et al.	
	043	US-7941279	05-10-2011	HWANG; Jenq-Neng et al.	
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	059	US-9422593	08-23-2016	ROTHMANN; Thomas [De] et al.	
	060	US-9441266	09-13-2016	LARSON; Jonathan William et al.	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 4 of 27

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Application Number	
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First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	061	US-9447457	09-20-2016	CHUN; Jong Yoon [Kr] et al.	
	062	US-9458497	10-04-2016	HASSIBI; Arjang et al.	
	063	US-9791372	10-17-2017	MALIK; Imran R. et al.	
	064	US-10066263	09-04-2018	RAJAGOPAL; Aditya et al.	
	065	US-10068051	09-04-2018	KARTALOV; Emil et al.	
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	067	US-10301668	05-28-2019	JACKY; Lucian A.E.	
	068	US-10770170	09-08-2020	KARTALOV; Emil P. et al.	
	069	US-20020022273	02-21-2002	EMPEDOCLES; Stephen A. [Us] et al.	
	070	US-20020146734	10-10-2002	ORTYN; William E. et al.	
	071	US-20030134320	07-17-2003	BARRUS; Jeffrey K. et al.	
	072	US-20030148280	08-07-2003	HARRIS; Barbara J. et al.	
	073	US-20030148544	08-07-2003	NIE; Shuming et al.	
	074	US-20040053230	03-18-2004	SCHAFFER; Michael Edward [Us] et al.	
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	076	US-20050053950	03-10-2005	ZUDAIRE UBANI; Enrique et al.	
	077	US-20050064435	03-24-2005	SU; Xing et al.	
	078	US-20050106607	05-19-2005	YIN; Li-Te et al.	
	079	US-20050164264	07-28-2005	SHIPWASH; Edward	
	080	US-20050214753	09-29-2005	SHULTZ; John W. et al.	

Examiner Signature		Date Considered	

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Sheet 5 of 27

Complete if Known	
Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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

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Complete if Known	
Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	102	US-20100233686	09-16-2010	HIGUCHI; Russell et al.	
	103	US-20100248257	09-30-2010	JACOBSEN; Kivin et al.	
	104	US-20100261026	10-14-2010	FERREE; Sean M. et al.	
	105	US-20100267064	10-21-2010	KARTALOV; Emil P. et al.	
	106	US-20100273173	10-28-2010	HIRAI; Mitsuharu et al.	
	107	US-20100324834	12-23-2010	TREPTOW; Rainer [De] et al.	
	108	US-20110104684	05-05-2011	HOOPER; Dennis G.	
	109	US-20110151459	06-23-2011	ROTHMANN; Thomas et al.	
	110	US-20110151550	06-23-2011	SAGNER; Gregor et al.	
	111	US-20110171658	07-14-2011	CARRICK; James M.	
	112	US-20110207623	08-25-2011	DIMITROV; Krassen et al.	
	113	US-20110223602	09-15-2011	WHITMAN; Douglas F. [Us] et al.	
	114	US-20110237459	09-29-2011	NOVA; Michael P. et al.	
	115	US-20120003646	01-05-2012	JOO; Sunghae A. et al.	
	116	US-20120040349	02-16-2012	VON; Lode Piia et al.	
	117	US-20120040352	02-16-2012	WANGH; Lawrence J. [Us] et al.	
	118	US-20120045756	02-23-2012	ROTHMANN; Thomas et al.	
	119	US-20120077195	03-29-2012	LI; Qingge et al.	
	120	US-20120101740	04-26-2012	ORPANA; Arto [Se] et al.	
	121	US-20120122704	05-17-2012	ATCHLEY; Daniel H. et al.	

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Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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	122	US-20120141995	06-07-2012	LI; Qingge et al.	
	123	US-20120171677	07-05-2012	LUDOWISE; Peter D.	
	124	US-20120184017	07-19-2012	CHATTERJEE; DEB K.	
	125	US-20120190030	07-26-2012	CHUN; Jong Yoon et al.	
	126	US-20120196283	08-02-2012	BABIEL; Reiner et al.	
	127	US-20120244534	09-27-2012	CHING; Jesus et al.	
	128	US-20120252014	10-04-2012	LOEFFERT; Dirk et al.	
	129	US-20120252017	10-04-2012	REED; Michael W. et al.	
	130	US-20120258457	10-11-2012	JAROSCH; Florian et al.	
	131	US-20120302448	11-29-2012	HUTCHISON; John Brian et al.	
	132	US-20130017971	01-17-2013	GEISS; Gary K. [Us] et al.	
	133	US-20130040841	02-14-2013	SAXONOV; Serge et al.	
	134	US-20130078626	03-28-2013	WASSERSTROM; Adam et al.	
	135	US-20130116780	05-09-2013	MILLER; Eran et al.	
	136	US-20130261019	10-03-2013	LIN; Chenxiang [Us] et al.	
	137	US-20130288244	10-31-2013	DECIU; Cosmin et al.	
	138	US-20140004520	01-02-2014	MOHAPATRA; Shyam S. et al.	
	139	US-20140038195	02-06-2014	MALIK; Imran R. [Us] et al.	
	140	US-20140171341	06-19-2014	JOUVENOT; Yann et al.	
	141	US-20140213471	07-31-2014	RAJAGOPAL; Aditya et al.	

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First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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

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Sheet 9 of 27

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Application Number	
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First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

FOREIGN PATENT DOCUMENTS

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

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Sheet 10 of 27

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Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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

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Sheet 11 of 27

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Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 12 of 27

Complete if Known	
Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	031	WO-2010128206-A1	11-11-2010	EXPRESSION ANALYTICS OY [FI], et al.		☐
	032	WO-2011047087-A2	04-21-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	033	WO-2011047087-A3	08-18-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	034	WO-2011100541-A2	08-18-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	035	WO-2011100541-A3	01-05-2012	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	036	WO-2011116088-A2	09-22-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	037	WO-2011116088-A3	02-02-2012	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	038	WO-2012058638-A2	05-03-2012	HARVARD COLLEGE [US], et al.		☐
	039	WO-2012058638-A3	12-20-2012	HARVARD COLLEGE [US], et al.		☐
	040	WO-2012106428-A2	08-09-2012	UNIV FLORIDA [US], et al.		☐

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

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	051	WO-2017218777-A1	12-21-2017	CALIFORNIA INST OF TECHN [US]		☐
	052	WO-2018081178-A1	05-03-2018	TWO PORE GUYS INC [US]		☐
	053	WO-2019006023-A1	01-03-2019	CHROMACODE INC [US]		☐
	054	WO-2019079204-A1	04-25-2019	CHROMACODE INC [US]		☐
	055	WO-2019169043-A1	09-06-2019	CHROMACODE INC [US]		☐
	056	WO-9746714-A1	12-11-1997	UNIV UTAH RES FOUND [US], et al.		☐
	057	WO-99/52708	10-21-1999	LUMINEX CORP [US]		☐
	058	WO-9919515-A1	04-22-1999	LUMINEX CORP [US]		☐

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

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Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

NON-PATENT LITERATURE DOCUMENTS

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

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

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	030	HENEGARIU, et al. Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques. 1997 Sep;23(3):504-11.	☐
	031	HIV databases. http://www.hiv.lanl.gov/content/index. Accessed February 2012.	☐
	032	Holland, et al., Detection of specific polymerase chain reaction product by utilizing the 5′ to 3′ exonuclease activity of Thermus aquaticus DNA polymerase. PNAS (USA) 88:7276–7280, 1991.	☐
	033	HOREJSH, et al. A molecular beacon, bead-based assay for the detection of nucleic acids by flow cytometry. Nucleic Acids Res. 2005 Jan 19;33(2):e13.	☐
	034	HUANG, et al. Identification of 8 foodborne pathogens by multicolor combinational probe coding technology in a single real-time PCR. Clin Chem. 2007 Oct;53(10):1741-8. Epub 2007 Aug 10.	☐
	035	HUANG, et al. Multicolor combinatorial probe coding for real-time PCR. PLoS One. 2011 Jan 14;6(1):e16033. .	☐
	036	HUDECOVA, I. et al. Maternal Plasma Fetal DNA Fractions in Pregnancies with Low and High Risks for Fetal Chromosomal Aneuploidies. PLoS ONE 9(2):e88484 (2014). .	☐
	037	International Search Report and Written Opinion dated 11/21/19 for PCT/US2019/050050.	☐
	038	JOTHIKUMAR, et al. Design of FRET-TaqMan probes for multiplex real-time PCR using an internal positive control. Biotechniques. 2009 Jun;46(7):519-24. .	☐
	039	KLOSTRANEC et al., Convergence of quantum dot barcodes with microfluidics and signal processing for multiplexed high-throughout infectious disease diagnostics. Nano Letters, 7(9):2812-2818, 2007.	☐

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	040	KUHN et al. Hybridization of DNA and PNA molecular beacons to single-stranded and double-stranded DNA targets. J Am Chem Soc. 124(6):1097-103 (2002).	☐
	041	LAO, et al. Multiplexing RT-PCR for the detection of multiple miRNA species in small samples. Biochem Biophys Res Commun. 2006 Apr 28;343(1):85-9. Epub 2006 Feb 28.	☐
	042	LEE, et al. Novel multiplex PCR using dual-priming oligonucleotides for detection and discrimination of the Mycobacterium tuberculosis complex and M. bovis BCG. J Clin Microbiol. 2010 Dec;48(12):4612-4. Epub 2010 Oct 13.	☐
	043	LEE, et al. Seven-color, homogeneous detection of six PCR products. Biotechniques. 1999 Aug;27(2):342-9.	☐
	044	LI et al., Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes. Nature Biotechnology, 23(7):885-889, 2005.	☐
	045	LIEW, et al. Validating a custom multiplex ELISA against individual commercial immunoassays using clinical samples. Biotechniques. 2007 Mar;42(3):327-8, 330-3.	☐
	046	LIN et al., Self-assembled combinatorial encoding nanoarrays for multiplexed biosensing. Nano Letters, 7(2):507-512, 2007.	☐
	047	LIVAK, et al. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. 1995 Jun;4(6):357-62.	☐
	048	LOFTIS et al., Principles of real-time PCR. Veterinary PCR Diagnostics pp.3-17 (2012).	☐
	049	LOGAN,et al., An Overview of real-time PCR platforms. 2004, In Real-time PCR: An essential guide, pg. 13-30.	☐

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Sheet 20 of 27

Complete if Known	
Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

NON-PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No.[1]	Include name of the author(in CAPITAL LETTERS),title of the article(when appropriate), title of the item (book,magazine,journal,serial,symposium,catalog,etc.),date,page(s),volume-issue number(s),publisher, city and/or country where published.	T[2]
	050	MORGAN et al., A commercial line probe assay for the rapid detection of rifampicin resistance in Mycobacterium tuberculosis: a systematic review and meta-analysis. BMC Infectious Diseases. 5:62 doi:10.1186/1471-2334-5-62 (2005).	☐
	051	MORRISON, et al. Two-color ratio-coding of chromosome targets in fluorescence in situ hybridization: quantitative analysis and reproducibility. Cytometry. 1997 Apr 1;27(4):314-26.	☐
	052	MYERS et al., A Handheld Point-of-Care Genomic Diagnostic System. PLoS One 8(8): e70266; pages 1-9 (2013).	☐
	053	NAVARRO et al., Real-time PCR detection chemistry. Clin Chim Acta. 439:231-250 (2015).	☐
	054	NOORDHOEK, et al. Sensitivity and specificity of PCR for detection of Mycobacterium tuberculosis: a blind comparison study among seven laboratories. J Clin Microbiol. 1994 Feb;32(2):277-84.	☐
	055	OLIVEIRA, et al. Multiplex PCR strategy for rapid identification of structural types and variants of the mec element in methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother. 2002 Jul;46(7):2155-61.	☐
	056	OU, et al. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science. 1988 Jan 15;239(4837):295-7.	☐
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First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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

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Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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	072	PCT/US2019/035129 Invitation to Pay Additional Fees dated August 19, 2019.	☐
	073	PCT/US2019/040392 International Preliminary Report on Patentability dated January 05, 2021.	☐
	074	PCT/US2019/041239 International Preliminary Report on Patentability dated January 21, 2021.	☐
	075	PCT/US2019/041239 International Search Report and Written Opinion dated September 30, 2019.	☐
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	079	PTAK, et al. Inhibition of human immunodeficiency virus type 1 replication in human cells by Debio-025, a novel cyclophilin binding agent. Antimicrob Agents Chemother. 2008 Apr;52(4):1302-17. Epub 2008 Jan 22.	☐
	080	RAJAGOPAL et al. Supercolor Coding Methods for Large-Scale Multiplexing of Biochemical Assays. Analytical Chemistry 85:7629-7636 (2013).	☐
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Sheet 23 of 27

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Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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	082	Rickert et al., "Multiplexed Real-Time PCR Using Universal Reporters," Clin. Chem., 50(9):1680- 1683, 2004.	☐
	083	ROSENSTRAUS, et al. An internal control for routine diagnostic PCR: design, properties, and effect on clinical performance. J Clin Microbiol. 1998 Jan;36(1):191-7.	☐
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	085	SAIKI, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, 239(4839):487-491 (January 29, 1988).	☐
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	087	SANGER et al. DNA sequencing with chain-terminating inhibitors. PNAS USA 1977 Dec;74(12):5463-7 (1977).	☐
	088	Second Office Action for Chinese Patent Application No. 201380049844.9 dated September 19, 2016 (w/translation).	☒
	089	SPEICHER, et al. Karyotyping human chromosomes by combinatorial multi-fluor FISH. Nat Genet. 1996 Apr;12(4):368-75.	☐
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	091	TIRASOPHON, et al. A novel detection of a single Plasmodium falciparum in infected blood. Biochem Biophys Res Commun. 1991 Feb 28;175(1):179-84.	☐

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Sheet 24 of 27

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Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

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

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

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	121	VOGELSTEIN, et al., Digital PCR, PNAS August 3, 1999 96 (16) 9236-9241; https://doi.org/10.1073/pnas.96.16.9236.	☐
	122	WANG, et al. Locked nucleic acid molecular beacons. J Am Chem Soc. 2005 Nov 16;127(45):15664-5.	☐
	123	WATERS, et al. Microchip device for cell lysis, multiplex PCR amplification, and electrophoretic sizing. Anal Chem. 1998 Jan 1;70(1):158-62.	☐
	124	WEIDMANN, et al. Rapid detection of herpes simplex virus and varicella-zoster virus infections by real-time PCR. J Clin Microbiol. 2003 Apr;41(4):1565-8.	☐
	125	WIESE, et al. Simultaneous multianalyte ELISA performed on a microarray platform. Clin Chem. 2001 Aug;47(8):1451-7.	☐
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	128	ZHANG, et al. A novel real-time quantitative PCR method using attached universal template probe. Nucleic Acids Res. 2003 Oct 15;31(20):e123(pp. 1-8).	☐

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Application Number	
Filing Date	2023-07-13
First Named Inventor	KARTALOV EMIL; P.
Art Unit	
Examiner Name	Not yet assigned
Attorney Docket Number	88DG-373535-US

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author(in CAPITAL LETTERS),title of the article(when appropriate), title of the item (book,magazine,journal,serial,symposium,catalog,etc.),date,page(s),volume-issue number(s),publisher, city and/or country where published.	T[2]
	129	ZHANG, et al. Novel Multiplex PCR Assay for Characterization and Concomitant Subtyping of Staphylococcal Cassette Chromosome mec Types I to V in Methicillin-Resistant Staphylococcus aureus. J Clin Microbiol. 2005 Oct;43(10):5026-33.	☐
	130	ZHAO et al. Comprehensive Algorithm for Quantitative Real-Time Polymerase Chain Reaction. Journal of Computational Biology 12(8):1047-1064 (2005).	☐

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. 1 Applicant's unique citation designation number (optional). 2 Applicant is to place a check mark here if English language Translation is attached. This collection of information is required by 37 CFR 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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

CERTIFICATION STATEMENT

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

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

OR

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

☐ See attached certification statement.

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

☒ A certification statement is not submitted herewith.

SIGNATURE

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

Signature	/Jesse A. Salen/	Date (YYYY-MM-DD)	2023-07-13
Name/Print	Jesse A. Salen	Registration Number	72209

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

Doc Code: TRACK1.REQ
Document Description: TrackOne Request

PTO/AIA/424 (04-14)

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

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

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

1. The processing fee set forth in 37 CFR 1.17(i)(1) and the prioritized examination fee set forth in 37 CFR 1.17(c) have been filed with the request. The publication fee requirement is met because that fee, set forth in 37 CFR 1.18(d), is currently $0. The basic filing fee, search fee, and examination fee are filed with the request or have been already been paid. I understand that any required excess claims fees or application size fee must be paid for the application.

2. I understand that the application may not contain, or be amended to contain, more than four independent claims, more than thirty total claims, or any multiple dependent claims, and that any request for an extension of time will cause an outstanding Track I request to be dismissed.

3. The applicable box is checked below:

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

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

---OR---

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

ii. An executed inventor's oath or declaration under 37 CFR 1.63 or 37 CFR 1.64 for each inventor, **or** the application data sheet meeting the conditions specified in 37 CFR 1.53(f)(3)(i) is filed with the application.

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

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

Signature /Jesse A. Salen/	Date July 13, 2023
Name (Print/Typed) Jesse A. Salen	Practitioner Registration Number 72209

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

☐ *Total of _____ forms are submitted.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION

Title:

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

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

Sheets of Drawings: 8

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

CROSS-REFERENCE TO RELATED APPLICATIONS

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

SEQUENCE LISTING

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

GOVERNMENT RIGHTS

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

BACKGROUND

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

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SUMMARY OF THE INVENTION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

INCORPORATION BY REFERENCE

[0045] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

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

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

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

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

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

[0051] Fig. 5 shows a schematic of two different methods of detecting wavelength and intensity of a light signal, such as a fluorescence emission signal.

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

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

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

DETAILED DESCRIPTION OF THE INVENTION

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

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

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

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

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

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

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

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

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

I. Definitions

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

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

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

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

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

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

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

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

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

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

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

II. Encoding and Decoding Methods

A. Traditional Fluorescent Encoding and Decoding Method

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

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

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

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

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

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

B. Encoding Methods Using More Than One Color Per Analyte

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

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

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

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

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

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

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

[0080] The coding scheme shown in Table 2 encodes each analyte using one control color and one additional color (up to 2 probes per analyte). However, as shown below, the number of analytes that can be encoded increases when up to 4 colors are used per analyte. Table 3 shows

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

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

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

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

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

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

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

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

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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

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

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

malfunctioned. Thus, the control (p) and the decoding matrix provide a means of verifying that the assay is functioning properly.

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

C. Reducing or Eliminating Degeneracy

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

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

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potential analyte reduces or eliminates degeneracy. For example, with reference to the coding scheme described in Table 3 and the decoding matrix described in Table 4 (enumerating every legitimate result), eliminating any two of analytes D, E, and F eliminates the degeneracy. Eliminating any one of analytes D, E, and F would not eliminate the degeneracy, but would reduce it.

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

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

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

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

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

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

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

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

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

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

[0095] Thus, in this context, the term "tier" is generally used to describe a set of codes that fully utilize the coding capability provided by a particular number of first values (e.g., intensities) and second values (e.g., colors), without degeneracy. For example, Tier 1 of Table 6 fully utilizes the coding capability provided by four colors with up to one intensity in each color, without degeneracy. As shown in Table 6, Tier 1, this results in six encoded analytes, including the control. To introduce a second tier, the minimum value of Y (described above) may be

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incremented to equal the cumulative maximum result from the prior tier(s) plus one (or more than one). In the example provided in Table 6, the intensity of the blue (control) color is maintained as one, to preserve the sequence counting capability in this color. Thus, Tier 2 consists of five non-degenerate encoding possibilities obtained by incrementing the intensities in the green, yellow, and red channels to equal the cumulative maximum results in each of these channels from Tier 1, plus one. All possibilities of these codes may then be enumerated for Tier 2, and codes resulting in degeneracy may be eliminated, or Tier 2 may be made non-degenerate by design, using the information used to eliminate the corresponding codes from Tier 1. Further coding capacity may then be achieved by adding a third tier, or further tiers, which are constructed according to analogous methods. A coding scheme may have an infinite number of tiers, for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100 or more tiers.

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

[0097] Continuing to refer to Table 6, a third tier (Tier 3) is constructed using the same principles described above. Analyte O is encoded by 1-0-0-16. The value of the control color is still maintained as 1. The value of the red color is equal to the cumulative maximum result from the prior tiers (15) plus 1, or 16. Similarly, analytes P and Q are encoded by 1-0-9-0 and 1-16-0-0, respectively. Combinations of these codes are used to encode the remaining four analytes (R-U), as was done for analytes D-G in Tier 1 and analytes K-N in Tier 2. In Tier 3, the inclusion of potential analytes R (1-0-9-16) and T (1-16-9-0) results in degeneracy. Therefore, these analytes have been eliminated from the coding scheme, to eliminate degeneracy.

[0098] The three-tier coding scheme shown in Table 6 shows the encoding of 15 analytes and one control using four colors. This coding scheme may be indefinitely extended, by adding more

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intensities to generate additional tiers and/or adding more colors, to generate additional coding capability within the tiers. The methods of reducing or eliminating degeneracy, as described in this disclosure, may be used with the addition of each intensity and/or color, to reduce or eliminate degenerate results.

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

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

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	B	1	0	1	0	
	C	1	1	0	0	
	~~**D**~~	~~1~~	~~0~~	~~1~~	~~1~~	Eliminated from coding scheme to eliminate degeneracy.
	E	1	1	0	1	
	~~**F**~~	~~1~~	~~1~~	~~1~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	G	1	1	1	1	
Cumulative Maximum Result		*6*	*3*	*2*	*3*	
2	**H**	1	0	0	4	
	I	1	0	3	0	
	J	1	4	0	0	
	~~**K**~~	~~1~~	~~0~~	~~3~~	~~4~~	Eliminated from coding scheme to eliminate degeneracy.
	L	1	4	0	4	
	~~**M**~~	~~1~~	~~4~~	~~3~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	N	1	4	3	4	
Cumulative Maximum Result		*11*	*15*	*8*	*15*	
3	**O**	1	0	0	16	
	P	1	0	9	0	
	Q	1	16	0	0	
	~~**R**~~	~~1~~	~~0~~	~~9~~	~~16~~	Eliminated from coding scheme to eliminate degeneracy.
	S	1	16	0	16	
	~~**T**~~	~~1~~	~~16~~	~~9~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	U	1	16	9	16	
Cumulative Maximum Result						
Cumulative Maximum Result for Three Non-Degenerate Tiers		***16***	***63***	***26***	***63***	

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

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

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

Tier	Analyte	B	G	Y	R	Comments
1	**Seq 1**	1	0	0	0	
	Seq 2	1	1	0	0	
	Seq 3	1	0	1	0	
	Seq 4	1	0	0	1	
	Seq 5	1	1	0	1	
	Seq 6	1	1	1	1	
	~~**Eliminated Potential Seq**~~	~~1~~	~~0~~	~~1~~	~~1~~	Eliminated from coding scheme to eliminate degeneracy.
	~~**Eliminated Potential Seq**~~	~~1~~	~~1~~	~~1~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.

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Cumulative Maximum Result		*6*	*3*	*2*	*3*	
2	**Seq 7**	1	4	0	0	
	Seq 8	1	0	3	0	
	Seq 9	1	0	0	4	
Etc. - i.e. Continue as provided in Table 6.						

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

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

[00104] Like the encoding method described in Table 6, this coding scheme is theoretically infinite, non-degenerate by construction, and only limited by the bandwidth of the instrument used to measure the signal. However, this coding scheme enables more analytes to be quantified per unit of bandwidth than the encoding method described in Table 6. The reason is that the available multiplicity of signal is used with maximal efficiency, as each level of intensity is utilized in the coding (i.e. there are no gaps in the chromatogram; see below for description of chromatograms). Table 8 shows one embodiment of this method, illustrating four tiers of encoding based on four colors and intensities 1, 2, 4, and 8. The coding scheme is non-degenerate, and the result if all 16 analytes are present is 15-15-15-15. This encoding method is more efficient, in terms of bandwidth utilization, than the encoding methods presented above. However, this method does not have the proofreading capability of the first scheme, as all the results decode to legitimate outcomes in the absence of gaps in the chromatogram.

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

Tier	Analyte	B	G	Y	R
1	**A**	1	0	0	0
	B	2	0	0	0
	C	4	0	0	0

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	D	8	0	0	0
2	**E**	0	1	0	0
	F	0	2	0	0
	G	0	4	0	0
	H	0	8	0	0
3	**I**	0	0	1	0
	J	0	0	2	0
	K	0	0	4	0
	L	0	0	8	0
4	**M**	0	0	0	1
	N	0	0	0	2
	O	0	0	0	4
	P	0	0	0	8
		15	***15***	***15***	***15***

[00105] The method presented above, and exemplified in Table 8, may be extended by introducing additional colors and/or intensities. For example, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more colors may be used. Additional intensity levels, such as 16, 32, 64, 128, 255, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, and so on may also be used. The method is generalizable and may be used to encode an infinite number of analytes. Each analyte is represented by a code in a single color, wherein the value of the code in that single color equal to the sum of all previous values plus one. For example, if the first code contains a 1 in a particular color, the next codes are 2, 4, 8, 16, 32, 64, 128 and so on. Other progressions are possible, but will not be as efficient in terms of usage of the bandwidth. However, one of ordinary skill in the art will recognize that less efficient use of bandwidth may be desirable when one wishes to maximize separation between values. Similarly, the value 1 could be excluded from the coding scheme, for example to maximize the difference in the intensity between the first encoded value and instrumental noise. For example, in some cases a 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

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be 2, 4, 6, 8, 10, 12, 14, 16, and so on. This approach generates uniform gaps in the possible cumulative results, allowing for higher tolerance to noise in comparison to an analyte code progression of 1, 2, 4, 8, 16, 32, and so on, and its corresponding cumulative result progression of 1, 2, 3, 4, 5, 6, 7, 8, and so on. In this example, the increased robustness in the measurement comes at a cost of a decrease in the number of available codes within a fixed bandwidth of multiplicity. For example, if only 63 multiplicity states can be measured reliably, the coding scheme starting with 1 offers 7 codes per color whereas the coding scheme starting with 2 offers 6 codes per color. If 4 colors are available, the former will offer 28 codes, while the latter will offer 24 codes. In summary, both the starting signal intensity and the progression (i.e., difference between intensity values) may be scaled in order to maximize the ability to distinguish over instrumental noise and maximize the differences between the intensity values, thereby enhancing the ability to distinguish between distinct experimental outcomes.

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

[00107] As described throughout this specification, any suitable value may be used for C or F. For example, C may be at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 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,

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1048576, and so on. As described elsewhere in this disclosure, both the initial starting value of *F* and the steps in its progression may also be varied.

III. Analytes

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

[00109] In some cases, an analyte may be a polynucleotide, such as DNA, RNA, peptide nucleic acids, and any hybrid thereof, where the polynucleotide contains any combination of deoxyribo- and/or ribo-nucleotides. Polynucleotides may be single stranded or double stranded, or contain portions of both double stranded or single stranded sequence. Polynucleotides may contain any combination of nucleotides or bases, including, for example, uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine, isoguanine and any nucleotide derivative thereof. As used herein, the term "nucleotide" may include nucleotides and nucleosides, as well as nucleoside and nucleotide analogs, and modified nucleotides, including both synthetic and naturally occurring species. Polynucleotides may be any suitable polynucleotide for which one or more reagents (or probes) as described herein may be produced, including but not limited to cDNA, mitochondrial DNA (mtDNA), messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), nuclear RNA (nRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small Cajal body-specific RNA (scaRNA), microRNA (miRNA), double stranded (dsRNA), ribozyme, riboswitch or viral RNA. Polynucleotides may be contained within any suitable vector, such as a plasmid, cosmid, fragment, chromosome, or genome.

[00110] Genomic DNA may be obtained from naturally occurring or genetically modified organisms or from artificially or synthetically created genomes. Analytes comprising genomic DNA may be obtained from any source and using any methods known in the art. For example, genomic DNA may be isolated with or without amplification. Amplification may include PCR

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amplification, multiple displacement amplification (MDA), rolling circle amplification and other amplification methods. Genomic DNA may also be obtained by cloning or recombinant methods, such as those involving plasmids and artificial chromosomes or other conventional methods (see Sambrook and Russell, *Molecular Cloning: A Laboratory Manual.*, cited *supra.*) Polynucleotides may be isolated using other methods known in the art, for example as disclosed in *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV) or *Molecular Cloning: A Laboratory Manual*. If the isolated polynucleotide is an mRNA, it may be reverse transcribed into cDNA using conventional techniques, as described in Sambrook and Russell, *Molecular Cloning: A Laboratory Manual.*, cited *supra*.

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

[00112] An analyte may be any molecule from any pathogen, such as a virus, bacteria, parasite, fungus, or prion (e.g., PrP^{Sc}). Examples of viruses include those from the families Adenoviridae, Flaviviridae, Hepadnaviridae, Herpesviridae, Orthomyxoviridae, Papovaviridae, Paramyxoviridae, Picornaviridae, Polyomavirus, Retroviridae, Rhabdoviridae, and Togaviridae. Specific examples of viruses include adenovirus, astrovirus, bocavirus, BK virus, coxsackievirus, cytomegalovirus, dengue virus, Ebola virus, enterovirus, Epstein-Barr virus, feline leukemia virus, hepatitis virus, hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis D virus, hepatitis E virus, herpes simplex virus (HSV), HSV type 1, HSV type 2, human immunodeficiency virus (HIV), HIV type 1, HIV type 2, human papilloma virus (HPV), HPV type 1, HPV type 2, HPV type 3, HPV type 4, HPV type 6, HPV type 10, HPV type 11, HPV 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

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virus, Norwalk virus, parovirus, polio virus, rabies virus, respiratory syncytial virus, retrovirus, rhinovirus, rotavirus, Rubella virus, smallpox virus, vaccinia virus, West Nile virus, and yellow fever virus.

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

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

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

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

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

[00117] An analyte may be obtained from any suitable location, including from organisms, whole cells, cell preparations and cell-free compositions from any organism, tissue, cell, or environment. Analytes may be obtained from environmental samples, biopsies, aspirates, formalin fixed embedded tissues, air, agricultural samples, soil samples, petroleum samples, water samples, or dust samples. In some instances, an analyte may be obtained from bodily fluids which may include blood, urine, feces, serum, lymph, saliva, mucosal secretions, perspiration, central nervous system fluid, vaginal fluid, or semen. Analytes may also be obtained from manufactured products, such as cosmetics, foods, personal care products, and the like. Analytes may be the products of experimental manipulation including, recombinant cloning, polynucleotide amplification, polymerase chain reaction (PCR) amplification, purification methods (such as purification of genomic DNA or RNA), and synthesis reactions.

[00118] More than one type of analyte may be detected in each multiplexed assay. For example, a polynucleotide, a protein, a polypeptide, a lipid, a carbohydrate, a sugar, a small

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molecule, or any other suitable molecule may be detected simultaneously in the same multiplexed assay with the use of suitable reagents. Any combination of analytes may be detected at the same time.

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

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

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

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

IV. Signals

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

[00123] The methods presented in this disclosure may also utilize the measurement of a signal in at least two dimensions, also referred to as the measurement of at least two components of a signal. In comparison to the coding scheme described in the paragraph above, which relies on, for example, signal intensity to differentiate between analytes, utilization of at least two components of a signal (e.g., color and intensity) allows the generation of more unique codes per unit of signal intensity bandwidth. When at least two components of a signal are utilized, a cumulative measurement of the at least two components may be obtained for a single sample volume. For example in the coding scheme described in Table 5, the presence of each analyte results in a particular intensity (one component of the signal) in each of the four colors (a second

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component of a signal). The combination of these constituent signals leads to a cumulative signal that may be measured by measuring an intensity at each wavelength or range of wavelengths. The corresponding coding scheme or decoding matrix may then be used to convert the cumulative measurement into a determination of the presence or absence of an analyte.

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

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

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

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fluorescent detection is used). The methods of the invention are therefore applicable. The methods of the invention may also be used with chemiluminescent signals and electrochemical signals.

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

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

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

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

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

[00132] The number of analytes that can be encoded may be further expanded by utilizing additional second values (e.g., wavelengths) that are values or ranges of values from a second component of a signal. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19,

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20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second values that are values or ranges of values from a second component of a signal may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more second values that are values or ranges of values from a second component of a signal may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 second values that are values or ranges of values from a second component of a signal may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 second values that are values or ranges of values from a second component of a signal may be used.

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

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

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

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

[00137] More generally a cumulative measurement may be obtained for any quantifiable component of a signal, and for any quantifiable component of a signal at another quantifiable component of a signal. For example, at least a first component of a signal may be measured at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second components of a signal. At lest a first component of a signal may be measured at at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second components of a signal. At least a first component of a signal may be measured at less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30,

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31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 second components of a signal.

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

A. Probes, Primers, Fluorophores, and Quenchers

[00139] Some of the methods provided in this disclosure utilize a reagent that generates a signal in the presence of an analyte. Any suitable reagent may be used with the invention. Generally, a reagent will have an analyte-specific component and a component that generates a signal in the presence of the analyte. In some cases, these reagents are referred to as probes. The probes may be hybridization probes. The hybridization probes may be an oligonucleotide probe attached to a fluorophore and a quencher (e.g., a TAQMAN probe).

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

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

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

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

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analyte present. If no analyte is present, the probe will not hybridize to an analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be produced.

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

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

[00145] Attachment of probes and quenchers to a probe may be performed in the same reaction or in serial reactions. A series of reactions may be performed to label probes with at least one fluorophore and the reaction products may be mixed to generate a mixture of probes with different fluorophores.

[00146] If an oligonucleotide probe comprises two or more fluorophores, these fluorophores may be arranged to allow Förster (Fluorescence) resonance energy transfer (FRET) to occur between the fluorophores. Briefly, FRET is a mechanism of energy transfer between fluorophores. Using FRET-based probes allows one excitation source to generate two different fluorescence emission signals by excitation of a single fluorophore. For example, the methods described herein may used paired probes, where one probe in the pair is attached to a fluorophore

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and a quencher and a second probe is attached to two fluorophores (in close enough proximity for FRET to occur) and a quencher. Any combination of fluorophores and quenchers that provide for FRET may be used. Such an approach doubles the number of fluorescent probes that may be used with an excitation source, because a single excitation source can be used to produce two signals: one from the probe with one fluorophore and one from the probe with two fluorophores in close enough proximity to provide for FRET. For example, using the encoding method described in Table 8, the number of unambiguously detectable analytes could be increased from 16 to 32 by pairing each of the probes in Table 8 with a corresponding FRET probe.

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

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

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21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 hybridization probes.

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

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

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

[00152] In embodiments of the invention that utilize fluorescent labels, any suitable fluorescent label may be used. Exemplary fluorescent labels suitable for use with the invention

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

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

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

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another example, lyophilized reagents comprise PCR primers. In yet another example, lyophilized reagents comprise reagents suitable for performing a PCR reaction.

V. Analytical Techniques and Instrumentation

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

[00156] **Fig. 5** shows two examples of instrumental configurations that may be used to obtain cumulative measurements useful for the methods of the invention. With reference to **Fig. 5**, an instrument with a detector configured to detect both wavelength and intensity (e.g., a fluorometer) is shown in **501.** With reference to **501**, at least one excitation light source **502** is directed into a chamber **503** containing analytes and reagents that generate a signal in the presence of the analytes **504.** As described elsewhere, the analyte may be a nucleic acid and the reagents generating the signal may be hybridization probes comprising a fluorophore and a quencher. If the analyte is present, an emission signal **505** is generated. In the configuration shown in **501**, the wavelength and intensity of this emission signal are measured across a spectrum by a detector capable of generating a fluorescence emission spectrum **506.**

[00157] The wavelength and intensity may also be determined using a combination of a photodetector and band pass filters. This configuration is used in several thermal cyclers known in the art. With reference to **Fig. 5**, an instrument with band pass filters and a photodetector is depicted in **507.** With reference to **507**, at least one excitation light source **508** is directed into a

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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.**

[00158] The methods described in this disclosure are compatible with a variety of amplification methods, including quantitative PCR (qPCR) methods, end point PCR methods, reverse transcriptase PCR, and digital PCR methods. Digital PCR methods (e.g., DROPLET DIGITAL (BIORAD) and DYNAMIC ARRAY (FLUIDIGM)) produce highly sensitive quantification of polynucleotide copy numbers. The methods provided herein can be easily integrated into these systems to significantly expand their throughput by allowing multiplexing in a droplet or a dynamic array.

[00159] Similarly, the methods described in this disclosure may be applied in a real-time PCR assay. For example, real-time data may be fully recorded as the PCR runs to completion. The end-point values may then be decoded to indicate the presence or absence of analytes. The individual cycle thresholds (Ct) values may be analyzed for each color by detecting the maxima of the second derivative of the real-time curve in that color. As a particular analyte is amplified,

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fluorophores are released from its corresponding probes in the same ratios as the color multiplicities in the coding of that sequence. Hence, e.g. a code of 1100 will have the same Ct in blue and green, while a code of 1400 will have its green Ct precede its blue Ct by 2 cycles. This additional data enables determination of the identity and starting quantity of each analyte.

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

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

[00162] In some cases, the methods described in this disclosure utilize end-point PCR methods. However, in some cases a result may be obtained before the end-point. This may be advantageous when, for example, results are needed as soon as possible. For example, in one case calibration experiments are performed in which different starting concentrations of an analyte are analyzed to determine the cycle number at which the signal becomes saturated, as a function of the starting amount of the analyte. Using this data, a computer monitoring a PCR reaction in real-time can be programmed to search for saturation up to the maximal cycle number according to the limit of detection (LOD) of the particular system. If there is no amplification of

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an analyte (e.g., other than a positive control, if present), by the maximal cycle number, the result for that analyte is negative. If there is amplification of the analyte by the maximal cycle number, the result for that analyte is positive and the saturation intensity in each color may be used to decode the result using the coding scheme. In both cases, there would be no need to run the PCR reaction beyond the number of cycles set by the LOD for that instrument. Although PCR has been used to illustrate this quantitative method, one of ordinary skill in the art will readily recognize that similar principles could be applied in any catalytic system where the starting amount of an analyte limits the rate of reaction, and the rate of reaction can be measured and reported by an evolution in a signal intensity over time.

VI. Compositions and Kits

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

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

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

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

[00167] **Fig. 6** shows schematics of exemplary kits of the invention. With reference to **Fig. 6**, a kit comprising a kit body **601** is shown that contains reagents for the detection of analytes (e.g., polynucleotide probes comprising a fluorophore and a quencher) **602** and primers for the amplification of a nucleic acid **603.** The kit may also contain any other reagents, such as reagents suitable for performing an amplification reaction **604.** In the embodiment depicted in **601**, the reagents for the detection of analytes and the primers are each contained in a single volume (e.g., tube or a bottle). However, these reagents may also be provided in separate volumes, as depicted the kit body shown in **605.** The kit body **605** contains seven separate volumes. Three volumes (**606, 607,** and **608**) contain reagents for the detection of analytes (or mixtures of such reagents) and three volumes (**609, 610,** and **611**) contain primers (or mixtures of primers). The kit may also contain reagents suitable for performing an amplification reaction **604.**

[00168] **Fig. 6** is provided for illustrative purposes only. Any number of reagents for the detection of analytes (e.g., probes) and any number of primers may be included in a single tube or bottle, as appropriate for the application. For example, in some embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more reagents for the detection

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

VII. Systems and Software

[00169] The methods of this disclosure may also be performed as part of a system. For example, one or more steps of the methods described herein may be performed by software contained on a computer readable medium. The software may be used to encode analytes, check for degeneracy, reduce or eliminate degeneracy, and/or produce a decoding matrix. The software may also be used to convert cumulative measurements made on an instrument (e.g., a PCR machine) into information concerning the presence or absence of an analyte. For example, with reference to **Fig. 4**, a computer **401** may be connected to an input device **403**, a display **402**, and a printer **404**. The computer may execute one or more of the steps of any of the methods described in this disclosure, including, for example, encoding methods **410**, analysis methods **420**, and decoding methods **430.** A computer may reduce or eliminate degeneracy, for example as part of the encoding methods **410.** Similarly, a computer may generate a coding schme that is non-degenerate by design using, for example, mathematical iteration. A computer may also be connected to a network **405**. The network may be a local network and/or the Internet. The network may be used to perform the methods of the invention on multiple computers in different

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locations. For example, one computer may perform the encoding, another computer may perform the measuring, and yet another computer may perform the decoding. A computer may perform any or all of these methods. The network may be used to transmit information obtained using the methods described in this disclosure throughout the world, for example across a border.

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

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

VIII. Services

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

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customer or party may be any suitable customer or party with a need or desire to use the methods, systems, compositions, and kits of the invention.

EXAMPLES

Example 1: General Materials & Methods

1. General Reagents

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

2. DNA Sequences, Primers, and Probes

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

[00175] Two regions of diagnostic relevance from the HIV-1 genome, p17 and poly-protease, were selected from the Los Alamos National Laboratory HIV-1 reference sequence. A diagnostic sequence from the *Plasmodium falciparum* ChR7 gene was obtained from the UCSC *Plasmodium falciparum* Genome Browser. A sequence for Herpes Simplex Virus-2 was synthesized from the sequence obtained from the European Molecular Biology Library. Similarly a diagnostic sequence for the rpoB gene in *Mycobacterium tuberculosis* was synthesized from a sequence obtained from the European Molecular Biology Library. A PCR diagnostic sequence for Dengue Virus Type 3 was obtained from the National Institute of Health genetic sequence database.

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

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Table 9. HIV-1 polyprotease sequence information.

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

Table 10. HIV p17 sequence information.

Sequence Name	HIV-1 P17 199mer synthesized from bases 790 – 1186
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (3' to 5')	CAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATA TAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAG ACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAG AAAAAAGCACAGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGTCA
Forward Primer (3' to 5')	CAGCTACAACCATCCCTTCAGACA

Reverse Primer (3' to 5')	TGACCTGATTGCTGTGTCCTGTGT
Probe 1 (3' to 5')	**/56-FAM/**AGCAACCCTCTATTGTGTGCATCAAAGG/3BHQ_1
Probe 2 (3' to 5')	**/5Cy3/**AAAGCACAGCAAGCAGCAGCTGA/3BHQ_2/

Table 11. *Plasmodium* ChR7 sequence information.

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

Table 12. Herpes simplex virus 2 sequence information.

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

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

Table 13. *Mycobacterium tuberculosis* rpoB sequence information

Sequence Name	Mycobacterium Tuberculosis rpoB 200mer synthesized from Mycobacterium tuberculosis rpoB genome
Source	EMBL Bank GQ395623
Target sequence (3' to 5')	GAGTGCAAAGACAAGGACATGACGTACGCGGCCCCGCTGTTCGTCACGGC CGAGTTCATCAACAACAACACCGGCGAGATCAAGAGCCAGACGGTGTTCA TGGGTGACTTCCCGATGATGACCGAGAAGGGCACCTTCATCATCAACGGC ACCGAGCGCGTCGTGGTCAGCCAGCTGGTCCGCTCGCCCGGTGTGTACTT
Forward Primer (3' to 5')	GAGTGCAAAGACAAGGACATGACG
Reverse Primer (3' to 5')	AAGTACACACCGGGCGAGC

Probe 1 (3' to 5')	**/56-FAM/**CGCTGTTCGTCACGGCCGAGTTCAT/3BHQ_1/
Probe 2 (3' to 5')	**/5Cy3/**AGATCAAGAGCCAGACGGTGTTCATG/3BHQ_2/
Probe 3 (3' to 5')	**/5Cy5/**AAGGGCACCTTCATCATCAACGGCA/3IAbRQSp/

Table 14. Dengue virus type 3 sequence information.

Sequence Name	Dengue Virus Type 3 200mer synthesized from Dengue Virus Type 3 genome
Source	GenBank M93130
Target sequence (3' to 5')	ATGCCAACTGTGATTGAGCACTTAGAAAGACTACAAAGGAAACATGGAGG AATGCTTGTGAGAAATCCACTCTCACGAAACTCCACGCACGAAATGTATT GGATATCCAATGGTACAGGCAACATCGTCTCATTCACAATGACACACAGG AGACCCACCATAGAGAAAGATGTGGATTTAGGAGCAGGAACCCGACATGT
Forward Primer (3' to 5')	ATGCCAACTGTGATTGAGCACT
Reverse Primer (3' to 5')	ACATGTCGGGTTCCTGCTCCTAAA
Probe 1 (3' to 5')	**/56-FAM/**ACAAAGGAAACATGGAGGAATGCTTGTGA/3BHQ_1/
Probe 2 (3' to 5')	**/5Cy3/**ACTCTCACGAAACTCCACGCACGAAA/3BHQ_2/
Probe 3 (3' to 5')	**/56-ROXN/**ACAATGACACACAGGAGACCCACCAT/3BHQ_2/
Probe 4 (3' to 5')	**/5Cy5/**GGATATCCAATGGTACAGGCAACATCGT/3IAbRQSp/

3. Polymerase Chain Reactions

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

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

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

Table 15. Reagents for 14 PCR reactions.

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

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		Taq 5x Master Mix	-	42 µL
	Template	Malaria Template	15 nM	2 µL
	Primers	Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
	Probes	Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
4		UltraPure Water	-	26 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
	Probes	Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
5		UltraPure Water	-	32 µL
		Taq 5x Master Mix	-	42 µL
	Template	Herpes Template	17 nM	2 µL
	Primers	Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
	Probes	Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
6		UltraPure Water	-	26 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
	Probes	Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
7		UltraPure Water	-	30 µL
		Taq 5x Master Mix	-	42 µL
	Template	TB Template	16 nM	2 µL
	Primers	TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
8		UltraPure Water	-	30 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL

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		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
9		UltraPure Water	-	28 µL
		Taq 5x Master Mix	-	42 µL
	Template	Dengue Virus Template	17 nM	2 µL
	Primers	Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
10		UltraPure Water	-	22 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
11		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease RVS Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL

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

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

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

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

Table 16. Coding scheme for detection of pathogens.

	FAM	**Cy3**	**ROX**	**Cy5**
HIV poly-protease	1	0	0	0
HIV p17	1	1	0	0

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Plasmodium	1	0	1	0
HSV	1	0	0	1
Mycobacterium	1	1	0	1
Dengue Virus	1	1	1	1

[00181] To construct the chromatogram, the signals of the positive controls (reactions 1, 3, 5, 7, and 9) were used to assemble every possible combination of present sequences, in each color. The cumulative signals were plotted in a diagram, called a "chromatogram" (**Fig. 3**). Cumulative signals corresponding to all possible combinations of the same rank in the same color were organized into their own "band." Doing this for all four colors produced a level and band structure, against which the experimental results of each combination (reactions 11, 12, 13, and 14) could be evaluated. **Fig. 3** shows three replicates of a chromatogram constructed using the positive control samples, with experimental results 4112, 3121, and 3102 superimposed on the chromatogram. The experimental results are indicated by black dots.

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

[00183] Using these criteria, the output of Experiment 12 was designated 4112, which was the correct answer. The output of Experiment 14 could have been designated as 2121 or 3121, but 2121 is illegitimate, so the result must be 3121, which is the correct answer. The output of Experiment 13 could be designated as 3102 or 3101, both of which are legitimate, but the result

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is clearly closer to 2 than 1 in the Cy5 channel, so 3102, the correct answer, was designated as the result. These results provide experimental validation of the methods of the provided herein.

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

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

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

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

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than one, for example to better distinguish over noise. Similarly, the values assigned to each analyte can be greater than the cumulative intensity values for the prior analytes plus one, for example, to increase the separation between the intensities and enable more simple assignment of intensities.

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

	FAM
Dengue Virus	1
HIV p17	2
HIV polyprotease	4

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

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

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

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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

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

Table 22. Binary FAM Experiment 5 Cocktail: dengue virus

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

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Dengue Virus Probe 1	200nM	4µL

Table 23. Binary FAM Experiment 6 Cocktail: HIV p17

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

Table 24. Binary FAM Experiment 7 Cocktail: HIV polyprotease

Reagents	Concentration	Volume Added
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	4µL
P17 Template	15nM	-

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

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

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

[00190] As expected, the intensity values for the combinations of analytes are equivalent to the sum of the assigned intensities. Since this coding scheme is designed to be non-degenerate, each result can be unambiguously decoded into the presence or absence of a

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particular analyte. For example, starting from the left side of **Fig. 7**, a result of 7 indicates the presence of all three analytes. A result of 3 indicates the presence of dengue virus and HIV p17 analytes, and the absence of HIV polyprotease. A result of 5 indicates the presence of dengue virus and HIV polyprotease, and the absence of HIV p17. Finally a result of 6 indicates the presence of HIV p17 and HIV polyprotease, and the absence of dengue virus. This coding scheme can be extended to encode additional analytes, as described elsewhere in this disclosure.

Supplemental Tables and Figures

Table S1 HIV-1 Poly Protease Sequence Information

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

Table S2 HIV-1 P17 Sequence Information

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

Table S3 Malaria ChR7 Sequence Information

Sequence Name	Malaria ChR7 199mer synthesized from bases 1139138 - 1141223
Source	UCSC Plasmodium falciparum Genome Browser Gateway

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Target sequence (5' to 3')	GCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAG AGGGAGCCTGAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTA AATTACCCAATTCTAAAGAAGAGAGGTAGTGACAAGAAATAACAATGCAA GGCCAATTTAAAACCTTCCCAGAGTAACAATTGGAGGGCAAGTCTGGTG
Forward Primer (5' to 3')	GCCTAACATGGCTATGACGGGTAA
Reverse Primer (5' to 3')	CACCAGACTTGCCCTCCAATTGTT
Probe 1 (5' to 3')	/56-FAM/ATTCCGGAGAGGGAGCCTGAGAAATA/3BHQ_1/
Probe 2 (5' to 3')	/56-ROXN/AAGGAAGGCAGCAGGCGCGTAAATTA/3BHQ_2/

Table S4 Herpes Simplex Virus-2 Sequence Information

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

Table S5 Tuberculosis rpoB Sequence Information

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

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Table S6 Dengue Virus Type 3 Sequence Information

Sequence Name	Dengue Virus Type 3 200mer synthesized from Dengue Virus Type 3 genome
Source	GenBank M93130
Target sequence (5' to 3')	ATGCCAACTGTGATTGAGCACTTAGAAAGACTACAAAGGAAACATGGAGG AATGCTTGTGAGAAATCCACTCTCACGAAACTCCACGCACGAAATGTATT GGATATCCAATGGTACAGGCAACATCGTCTCATTCACAATGACACACAGG AGACCCACCATAGAGAAAGATGTGGATTTAGGAGCAGGAACCCGACATGT
Forward Primer (5' to 3')	ATGCCAACTGTGATTGAGCACT
Reverse Primer (5' to 3')	ACATGTCGGGTTCCTGCTCCTAAA
Probe 1 (5' to 3')	/56-FAM/ACAAAGGAAACATGGAGGAATGCTTGTGA/3BHQ_1/
Probe 2 (5' to 3')	/5Cy3/ACTCTCACGAAACTCCACGCACGAAA/3BHQ_2/
Probe 3 (5' to 3')	/56-ROXN/ACAATGACACACAGGAGACCCACCAT/3BHQ_2/
Probe 4 (5' to 3')	/5Cy5/GGATATCCAATGGTACAGGCAACATCGT/3IAbRQSp/

Table S7 Experiment 1 Cocktail

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

Table S8 Experiment 2 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	28µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Malaria Template	15nM	2µL
Herpes Template	17nM	2µL
TB Template	16nM	2µL

Dengue Virus Template	17nM	2µL
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	2µL
Poly Protease RVS Primer	1µM	2µL
Probes	Concentration	Volume Added
Poly Protease Probe 1	1µM	2µL

Table S9 Experiment 3 Cocktail

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

Table S10 Experiment 4 Cocktail

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

Table S11 Experiment 5 Cocktail

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

Table S12 Experiment 6 Cocktail

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

Table S13 Experiment 7 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	30µL
Taq 5x Master Mix	-	42µL
Templates	Concentration	Volume Added
TB Template	16nM	2µL

Primers	Concentration	Volume Added
TB FWD Primer	1µM	2µL
TB RVS Primer	1µM	2µL
Probes	Concentration	Volume Added
TB Probe 1	1µM	2µL
TB Probe 2	1µM	2µL
TB Probe 3	1µM	2µL

Table S14 Experiment 8 Cocktail

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

Table S15 Experiment 9 Cocktail

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

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

Table S16 Experiment 10 Cocktail

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

Table S17 Experiment 11 Cocktail

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

Malaria RVS Primer	1μM	2μL
Herpes FWD Primer	1μM	2μL
Herpes RVS Primer	1μM	2μL
Dengue Virus FWD Primer	1μM	2μL
Dengue Virus RVS Primer	1μM	2μL
Probes	Concentration	Volume Added
Poly Protease Probe 1	1μM	2μL
Malaria Probe 1	1μM	2μL
Malaria Probe 2	1μM	2μL
Herpes Probe 1	1μM	2μL
Herpes Probe 2	1μM	2μL
Dengue Virus Probe 1	1μM	2μL
Dengue Virus Probe 2	1μM	2μL
Dengue Virus Probe 3	1μM	2μL
Dengue Virus Probe 4	1μM	2μL

Table S18 Experiment 12 Cocktail

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

Poly Protease Probe 1	1µM	2µL
Malaria Probe 1	1µM	2µL
Malaria Probe 2	1µM	2µL
Herpes Probe 1	1µM	2µL
Herpes Probe 2	1µM	2µL
TB Probe 1	1µM	2µL
TB Probe 2	1µM	2µL
TB Probe 3	1µM	2µL

Table S19 Experiment 13 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	12µL
Taq 5x Master Mix	-	42µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	2µL
Herpes Template	17nM	2µL
TB Template	16nM	2µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	2µL
Poly Protease RVS Primer	1µM	2µL
Herpes FWD Primer	1µM	2µL
Herpes RVS Primer	1µM	2µL
TB FWD Primer	1µM	2µL
TB RVS Primer	1µM	2µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	1µM	2µL
Herpes Probe 1	1µM	2µL
Herpes Probe 2	1µM	2µL
TB Probe 1	1µM	2µL
TB Probe 2	1µM	2µL
TB Probe 3	1µM	2µL

Table S20 Experiment 14 Cocktail

Reagents	Concentration	Volume Added
UltraPure Water	-	10µL

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

Table S21 Binary FAM Experiment 1 Cocktail

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

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

Table S22 Binary FAM Experiment 2 Cocktail

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

Table S23 Binary FAM Experiment 3 Cocktail

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

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

Table S24 Binary FAM Experiment 4 Cocktail

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

Table S25 Binary FAM Experiment 5 Cocktail

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

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

Table S26 Binary FAM Experiment 6 Cocktail

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

Table S27 Binary FAM Experiment 7 Cocktail

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

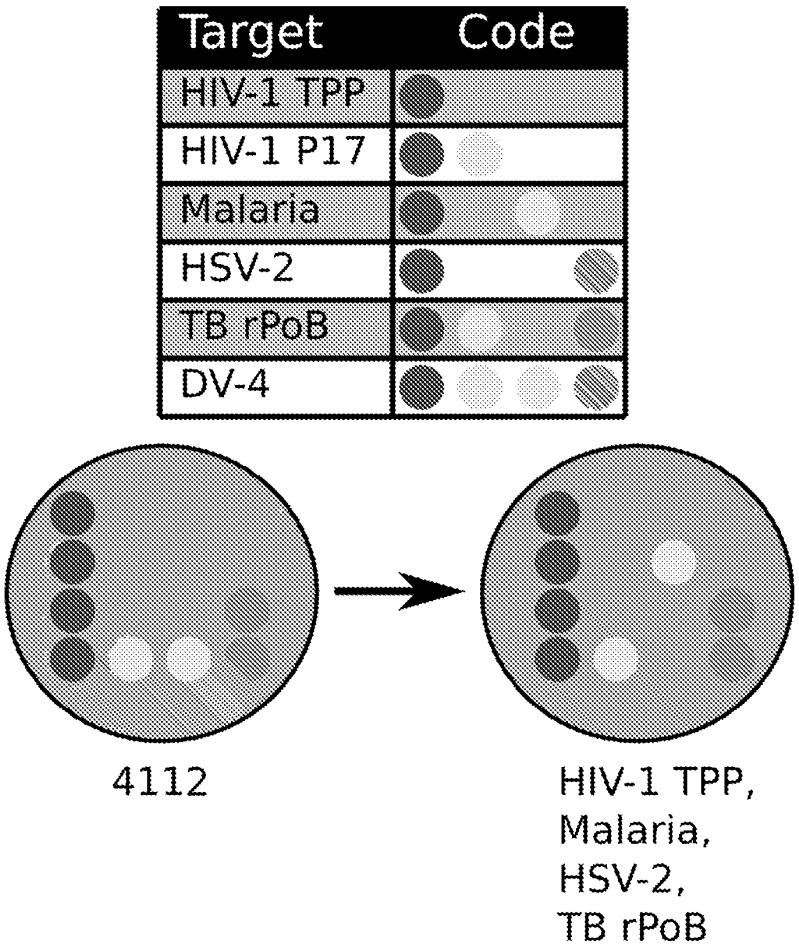


Figure S1. Readout of a valid code

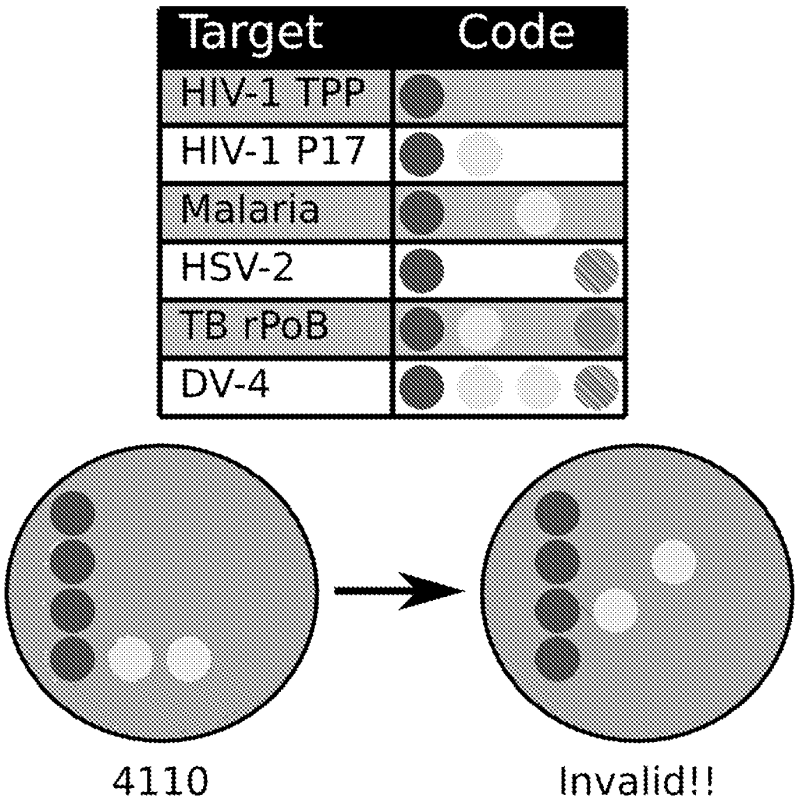


Figure S2. Readout of an invalid code

CLAIMS

WHAT IS CLAIMED IS:

1. A system comprising:

a sample chamber configured to house a sample and analyte-specific reagent mixtures of analyte-specific hybridization probes and multiple fluorophores;

a multi-channel detector to detect:

a first electromagnetic signal at a first wavelength from the sample chamber, the first electromagnetic signal generated by excitement of a first fluorophore of the multiple fluorophores;

a second electromagnetic signal at a second wavelength from the sample chamber, the second electromagnetic signal generated by excitement of a second fluorophore of the multiple fluorophores;

a third electromagnetic signal at a third wavelength from the sample chamber, the third electromagnetic signal generated by excitement of a third fluorophore of the multiple fluorophores;

a fourth electromagnetic signal at a fourth wavelength from the sample chamber, the fourth electromagnetic signal generated by excitement of a fourth fluorophore of the multiple fluorophores;

a processor controlled analyzer to receive, from the multi-channel detector, a cumulative signal based on the first, second, third, and fourth electromagnetic signals and apply a decoding matrix to the cumulative signal to unambiguously detect the presence or absence of each of *M* analytes by associating, for each analyte, a first value in a first component of the cumulative signal and a second value in a second component of the cumulative signal, wherein each first value is an intensity or range of intensities and each second value is a wavelength or a range of wavelengths, and wherein the second values comprise the first, second, third, and fourth wavelengths, and the determination is made without mass spectrometry or melting curve analysis; and

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wherein M is greater than the the number of the second values used to encode the analytes (C), the multi-channel detector comprises C channels, and M and C are positive integers.

2. The system of claim 1, wherein the multi-channel detector is further configured to detect:

a fifth electromagnetic signal at a fifth wavelength from the sample chamber, the fifth electromagnetic signal generated by excitement of a fifth fluorophore of the multiple fluorophores; and

a sixth electromagnetic signal at a sixth wavelength from the sample chamber, the sixth electromagnetic signal generated by excitement of a sixth fluorophore of the multiple fluorophores.

3. The system of claim 1, wherein $M=C*\log_2(F+1)$, wherein F is a positive integer and is equal to the maximum cumulative intensity of the first component of the signal, for any second value, when all of the analytes are present, and C=4, 5, or 6.

4. The system of claim 3, wherein F=3.

5. The system of claim 1, wherein the multiple fluorophores is selected from the group consisting of a blue fluorophore, a green fluorophore, a yellow fluorophore, a red fluorophore, and any combinations thereof.

6. The system of claim 1, wherein the multiple fluorophores is selected from the group consisting of a fluorescein amidite (FAM), a cyanine 3 (Cy3), a carboxy-X-rhodamine (ROX), a cyanine 5 (Cy5), a cyanine 5.5 (Cy5.5), and any combinations thereof.

7. The system of claim 1, wherein the multiple fluorophores has a maximum excitation wavelength selected from the group consisting of about 494 nm, about 550 nm, about 567 nm, about 650 nm, about 675 nm, and any combinations thereof.

8. The system of claim 1, wherein the multiple fluorophores has a maximum emission wavelength selected from the group consisting of about 518 nm, about 565 nm, about 591 nm, about 670 nm, about 697 nm, and any combinations thereof.

9. The system of claim 1, wherein the analyte-specific hybridization probes are labeled with a fluorophore of the multiple fluorophores.

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10. The system of claim 1, wherein the detector comprises four or six band pass filters.

11. The system of claim 1, wherein the detector comprises band pass filters that enable the detection of blue, green, and red fluorescent signals; and wherein the detector comprises band pass filters that enable the detection of ranges selected from the group consisting of from about 483 nm to about 533 nm, from about 523 nm to about 568 nm, from about 558 nm to about 610 nm, from about 615 nm to about 670 nm, and any combinations thereof.

12. The system of claim 1, wherein the multi-channel detector comprises four or six photodetectors.

13. The system of claim 1, wherein the multi-channel detector comprises photodetectors that enable the detection of blue, green, and red fluorescent signals.

14. The system of claim 1, wherein the multi-channel detector comprises photodetecors that enable the detection ranges selected from the group consisting of from about 483 nm to about 533 nm, from about 523 nm to about 568 nm, from about 558 nm to about 610 nm, from about 615 nm to about 670 nm, and any combinations thereof.

15. The system of claim 1, wherein the sample comprises a droplet.

16. The system of claim 15, wherein the droplet is housed in a dynamic array.

17. The system of claim 2, wherein each of the six fluorophores is attached to a hybridization probe.

18. The system of claim 1, further comprising a display coupled to the processor controlled analyzer to visualize a plot of the first and second values.

19. A system comprising:

a processor;

a display; and

a non-transitory computer readable medium storing instructions thereon that, when executed by the processor, cause the processor to:

obtain cumulative signal data from a digital PCR instrument with a sample volume, the digital PCR instrument comprising a light source and a multichannel detector with C channels, and the sample volume comprising M fluorescently labeled polynucleotide analytes;

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apply a decoding matrix to the cumulative signal data to unambiguously determine the presence or absence of each of the *M* fluorescently labeled polynucleotide analytes by associating, for each fluorescently labeled polynucleotide analyte, a first value in a first component of the cumulative signal data and a second value in a second component of the cumulative signal data, wherein each first value is an intensity or range of intensities and each second value is a wavelength or a range of wavelengths, wherein $M=C*\log_2(F+1)$, F is the maximum cumulative intensity of the first component of the cumulative signal data, for any second value, when all of the analytes are present, and *M*, *C*, and *F* are each positive integers; and

plot, on the display, a representation of the first value and the second value;

wherein the cumulative signal data comprises:

a first electromagnetic signal at a first wavelength from the sample volume, the first electromagnetic signal generated by excitement of a first fluorescently labeled polynucleotide analyte of the six fluorescently labeled polynucleotide analytes;

a second electromagnetic signal at a second wavelength from the sample volume, the second electromagnetic signal generated by excitement of a second fluorescently labeled polynucleotide analyte of the six fluorescently labeled polynucleotide analytes;

a third electromagnetic signal at a third wavelength from the sample volume, the third electromagnetic signal generated by excitement of a third fluorescently labeled polynucleotide analyte of the six fluorescently labeled polynucleotide analytes;

a fourth electromagnetic signal at a fourth wavelength from the sample volume, the fourth electromagnetic signal generated by excitement of a fourth first fluorescently labeled polynucleotide analyte of the six fluorescently labeled polynucleotide analytes; and

wherein the second values comprise the first, second, third, and fourth wavelengths, and the determination is made without mass spectrometry or melting curve analysis.

20. A reaction mixture for unambiguously detecting a presence or absence of a plurality of non-immobilized polynucleotide analytes of a single sample, the reaction mixture comprising:

a plurality of non-immobilized hybridization probes,

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wherein the reaction mixture is subjected to conditions such that the plurality of polynucleotide analytes are amplified;

wherein at least one of the plurality of oligonucleotide primers is used to amplify a region complementary of at least one of the plurality of hybridization probes;

wherein the plurality of hybridization probes comprise a first hybridization probe conjugated to a first fluorophore and a second fluorophore,

wherein the first fluorophore and the second fluorophore, when excited by a light source, emit light of different wavelengths, and

optionally the plurality of hybridization probes further comprises a third hybridization probe and a fourth hybridization probe, wherein the third hybridization probe is conjugated to a third fluorophore and the fourth hybridization is conjugated to a fourth fluorophore,

wherein the third fluorophore and the fourth fluorophore, when excited by a light source, emit light of a same wavelength, and

wherein the third hybridization probe and the fourth hybridization probe are present at different concentrations in the reaction mixture;

optionally the plurality of hybridization probes further comprises a fifth hybridization probe and a sixth hybridization probe, wherein the fifth hybridization probe is conjugated to a fifth fluorophore and the sixth hybridization is conjugated to a sixth fluorophore;

wherein the fifth fluorophore and the sixth fluorophore, when excited by a light source, emit light of a same wavelength, and

wherein the fifth hybridization probe and the six hybridization probe are present at different concentrations in the reaction mixture.

21. The reaction mixture of claim 20, wherein the reactions mixture is separated into droplets.

22. The reaction mixture of claim 20, wherein the first fluorophore, the second fluorophore, the third fluorophore or the fourth fluorophore is selected from the group consisting of a fluorescein amidite (FAM), a cyanine 3 (CY3), a carboxy-X-rhodamine (ROX), a cyanine 5 (CY5), a cyanine 5.5 (CY5.5), and any combinations thereof.

23. The reaction mixture of claim 20, wherein the first fluorophore, the second fluorophore, the third fluororphore or the fourth fluorophore has a maximum exitation

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wavelength selected from the group consisting of about 494 nm, about 550 nm, about 567 nm, about 650 nm, about 675 nm, and any combinations thereof.

24. The reaction mixture of claim 20, wherein the first fluorophore, the second fluorophore, the third fluororphore or the fourth fluorophore has a maximum emission wavelength selected from the group consisting of about 518 nm, about 565 nm, about 591 nm, about 670 nm, about 697 nm, and any combinations thereof.

25. The reaction mixture of claim 20, wherein a ratio of concentration between the first hybridization probe and the second hybridization probe is between 0.3 and 0.7 mol/mol.

26. The reaction mixture of claim 20, wherein the reaction mixture further comprises a polymerase having 5′ to 3′ exonuclease activity and primers specific for the plurality of polynucleotide analytes.

27. The reaction mixture of claim 24, wherein the first fluorophore, the second fluorophore, the third fluorophore, the fourth fluorophore, the fifth fluorophore, or the sixth fluorophore comprise multiple fluorophores.

28. The reaction mixture of claim 20, wherein one of the plurality of hybridization probes further comprise a fluorescent quencher.

29. The reaction mixture of claim 20, wherein the first hybridization probe and the second hybridization probe are present at different concentrations in the reaction mixture.

30. A kit for the unambiguously detecting a presence or absence of a plurality of polynucleotide analytes of a single sample comprising the components claim 20 and instructions for the use of the kit.

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ABSTRACT

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

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

Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

As the below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application, or

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

filed on February 1, 2013.

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

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

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

WARNING:

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LEGAL NAME OF INVENTOR

Inventor: Emil Kartalov Date (Optional): _____

Signature: Emil P. Kartalov

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

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LEGAL NAME OF INVENTOR

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

Signature: _____

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LEGAL NAME OF INVENTOR

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

Signature: Axel Scherer

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	88DG-373535-US
	Application Number	
Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
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☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

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Inventor 1 Remove

Legal Name

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

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

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Inventor 2 Remove

Legal Name

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Mailing Address of Inventor:

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Address 2			
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Postal Code	92865	Country	US

Inventor 3 Remove

Legal Name

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

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	88DG-373535-US
	Application Number	
Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

City	BARNARD	State/Province	VT	Country of Residence	US

Mailing Address of Inventor:

Address 1	1200 East California Boulevard		
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City	Pasadena	State/Province	CA
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Application Information:

Title of the Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS		
Attorney Docket Number	88DG-373535-US	Small Entity Status Claimed	☒
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	8	Suggested Figure for Publication (if any)	

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

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

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	88DG-373535-US
	Application Number	
Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

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Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	Continuation of	16937464	2020-07-23

Prior Application Status	Patented			Remove	
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
16937464	Continuation of	15914356	2018-03-07	10770170	

Prior Application Status	Patented			Remove	
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
15914356	Continuation of	14451876	2014-08-05	10068051	

Prior Application Status	Patented			Remove	
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
14451876	Continuation of	13756760	2013-02-01	8838394	

Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13756760	Claims benefit of provisional	61703093	2012-09-19

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	88DG-373535-US
	Application Number	
Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13756760	Claims benefit of provisional	61594480	2012-02-03
Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button.			Add

Foreign Priority Information:

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

			Remove
Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)
Additional Foreign Priority Data may be generated within this form by selecting the **Add** button.			Add

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

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

PTO/AIA/14 (11-15)
Approved for use through 04/30/2017. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	88DG-373535-US
	Application Number	
Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

Authorization or Opt-Out of Authorization to Permit Access:

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

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

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

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

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

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

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

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

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

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

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

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	88DG-373535-US
	Application Number	
Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

Applicant Information:

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

Applicant 1 Remove

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

Clear

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

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

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. [X]

Organization Name	CALIFORNIA INSTITUTE OF TECHNOLOGY		
Mailing Address Information For Applicant:			
Address 1	1200 East California Boulevard, MC 210-85		
Address 2			
City	Pasadena	**State/Province**	CA
Country	US	Postal Code	91125
Phone Number		Fax Number	
Email Address			

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

Assignee Information including Non-Applicant Assignee Information:

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

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Approved for use through 04/30/2017. OMB 0651-0032
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	88DG-373535-US
	Application Number	
Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

Assignee 1

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

Remove

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

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

Mailing Address Information For Assignee including Non-Applicant Assignee:

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

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

Signature:

Remove

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

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

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

Signature	/Jesse A. Salen/			Date (YYYY-MM-DD)	2023-07-13
First Name	Jesse A.	Last Name	Salen	Registration Number	72209

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

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Approved for use through 04/30/2017. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	88DG-373535-US
	Application Number	
Title of Invention	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

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

Privacy Act Statement

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

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

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

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

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

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

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

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

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

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

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

Doc Code - SEQ.XML

WIPO Type Code -

SLIC Placeholder Sheet for P-ELP (CMS) Content

Application Number: 18352112 Document Date: 07/13/2023

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

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist.

- Sequence Listing

At the time of document entry (noted above):

- USPTO employees may access CMS content via DAV or via the SLIC web page.
- External customers may access CMS content via PAIR/Patent Center Retrieval using the Supplemental Content tab.

Form Revision Date: 07 January 2022

Application Number: 18352112

Reviewed by: 97ClN/eHe4jhmn5GMimwpAP70alPRU2kNiDwvOWHBrv8bqgC4kdzMg==

Note: This validation report concerns the sequence listing filed 07-13-2023 18:22:52 and indicates that NO errors were found by the USPTO during validation of the sequence listing. This validation report should not be construed as meaning that the sequence listing complies with all of the requirements of 37 CFR §§ 1.821-1.825, 37 CFR §§ 1.831-1.835 and 1.839, WIPO Standard ST.25, or WIPO Standard ST.26.

For any sequence listing related questions or concerns, the Applicant is invited to contact the USPTO Sequence Listing Help Desk at 571-272-2510 or SequenceHelpDesk@USPTO.GOV.

Timestamp: [year=2023; month=7; day=13; hr=18; min=32; sec=38; ms=746]

Validated By CRFValidator v 1.5.0

Application No: 18352112 Version No: 1.1

CRF Copy Request from application Version :

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Output Set: /slic/CRF_WORKFLOW/18/352/112/1.1/report_18352112_1_1.xml.pdf

Started: 2023-07-13 18:27:05.000

Finished: 2023-07-13 18:27:17.241

Elapsed: 0 hr(s) 0 min(s) 12 sec(s) 241 ms

Total Warnings: 29

Total Errors: 0

No. of SeqIDs Defined: 32

Actual SeqID Count: 32

Error code	Error Description
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W 261033	DataElement:PROPERTY_NAMES.FEATURE_QUALS;DetectedValue:3"black hole quencher-1 modified nucleotide;MessageKey:X_FEATURE_QUALS_QUAL_NOTE_VAL_OTHER;LocalizedMessage:Verify that the "note" qualifier describes the "OTHER" value for the "mod_base" qualifier.; Sequence ID: 4
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Finished:	2023-07-13 18:27:17.241
Elapsed:	0 hr(s) 0 min(s) 12 sec(s) 241 ms
Total Warnings:	29
Total Errors:	0
No. of SeqIDs Defined:	32
Actual SeqID Count:	32

Error code	Error Description
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Finished: 2023-07-13 18:27:17.241

Elapsed: 0 hr(s) 0 min(s) 12 sec(s) 241 ms

Total Warnings: 29

Total Errors: 0

No. of SeqIDs Defined: 32

Actual SeqID Count: 32

Error code		Error Description
W	261033	DataElement:PROPERTY_NAMES.FEATURE_QUALS;DetectedValue:5"6-fluorescein amidite modified nucleotide;MessageKey:X_FEATURE_QUALS_QUAL_NOTE_VAL_OTHER;LocalizedMessage:Verify that the "note" qualifier describes the "OTHER" value for the "mod_base" qualifier.; Sequence ID: 23
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W	261178	DataElement:PROPERTY_NAMES.ORIGINAL_FREE_TEXT_LANGUAGE_CODE;MessageKey:ORIGINAL_FREE_TEXT_LANGUAGE_CODE_MISSING;LocalizedMessage:An original free text language code has not been entered.;

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Application Number: 18352112 Document Date: 07/13/2023

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