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*Attorneys for Plaintiff ChromaCode, Inc.*

UNITED STATES DISTRICT COURT

NORTHERN DISTRICT OF CALIFORNIA

CHROMACODE, INC.,

Plaintiff,

v.

BIO-RAD LABORATORIES, INC.,

Defendant.

CASE NO.:

**COMPLAINT FOR DECLARATORY JUDGMENT OF NON-INFRINGEMENT OF U.S. PATENT NOS. 9,222,128 AND 9,921,154**

DEMAND FOR JURY TRIAL

Plaintiff ChromaCode, Inc. ("ChromaCode") hereby seeks a declaratory judgment of non-infringement of United States Patent Nos. 9,222,128 and 9,921,154 as follows:

## NATURE OF THE ACTION

1. This is an action for a declaratory judgment of non-infringement arising under the patent laws of the United States, Title 35 of the United States Code. ChromaCode requests this relief because Defendant Bio-Rad Laboratories, Inc. ("Bio-Rad") claims that ChromaCode's "HDPCR Assays" infringe U.S. Patent Nos. 9,222,128 (the "'128 Patent") and 9,921,154 (the "'154 Patent") (collectively, the "Challenged Patents"). Bio-Rad's affirmative allegations of infringement of the Challenged Patents have created an immediate, real, and justiciable controversy between ChromaCode and Bio-Rad.

2. Neither ChromaCode nor its products practice the Challenged Patents. ChromaCode has not induced or contributed to any third-party product or technology that practices the Challenged Patents.

3. Nevertheless, on September 6, 2023, Bio-Rad sent ChromaCode a letter accusing ChromaCode of infringing the Challenged Patents and attaching exemplary infringement claim charts. As a result of Bio-Rad's letter and the impact of those claims on ChromaCode's business, there is an immediate, real, and justiciable controversy.

## PARTIES

4. ChromaCode is a Delaware corporation with its headquarters in Carlsbad, California. ChromaCode is a molecular diagnostics company with a bioinformatics focus.

5. Bio-Rad is a Delaware corporation with its headquarters in Hercules, California, which is within Contra Costa County.

## JURISDICTIONAL STATEMENT

6. This action arises under the Declaratory Judgment Act, 28 U.S.C. § 2201, and under the patent laws of the United States, 35 U.S.C. §§ 1-390.

7. This Court has subject matter jurisdiction over this action under 28 U.S.C. §§ 1331, 1338(a), and 2201(a). As detailed below, Bio-Rad's infringement allegations give rise

to an immediate, real, and justiciable controversy between ChromaCode and Bio-Rad as to whether ChromaCode is infringing or has infringed any claims of the Challenged Patents.

8. This Court has personal jurisdiction over Bio-Rad. Bio-Rad's headquarters are in California and its contacts with the state are so continuous and systematic as to render it essentially at home in California.

9. Venue is appropriate under 28 U.S.C. § 1400(b) because the Defendant Bio-Rad resides in the Northern District of California.

**INTRADISTRICT ASSIGNMENT**

10. For purposes of intradistrict assignment under Civil Local Rules 3-2(c) and 3-5(b), this Intellectual Property Action will be assigned on a district-wide basis.

**BACKGROUND**

11. On December 29, 2015, the USPTO issued the '128 Patent, entitled "Multiplexed digital assays with combinatorial use of signals" to Serge Saxonov, Simant Dube, Benjamin J. Hindson, and Adam M. McCoy. The face of the '128 Patent lists Bio-Rad as the assignee. A true and correct copy of the '128 Patent is attached hereto as **Exhibit A**.

12. On March 20, 2018, the USPTO issued the '154 Patent, entitled "Multiplexed digital assays" to Yann Jouvenot, Serge Saxonov, Simant Dube, and John Frederick Regan. The face of the '154 Patent lists Bio-Rad as the assignee. A true and correct copy of the '154 Patent is attached hereto as **Exhibit B**.

13. On September 6, 2023, Bio-Rad sent ChromaCode a letter accusing ChromaCode of infringing the Challenged Patents (the "Infringement Letter"). A true and correct copy of the Infringement Letter is attached hereto as **Exhibit C**.

14. The Infringement Letter, signed by Bio-Rad's Vice President and Assistant General Counsel, is titled "Notice of Potential Patent Infringement by ChromaCode's HDPCR Assays" and states the following:

> Specifically, based on the public information currently available to and reviewed by Bio-Rad, ChromaCode's activities—including the making, using, offering to sell, and selling of ChromaCode's HDPCR Assays—appear to infringe the following patents.

| | |
|---|---|
| U.S. Patent No. 9,222,128 ('128 Patent) | "MULTIPLEXED DIGITAL ASSAYS WITH COMBINATORIAL USE OF SIGNALS" |
| U.S. Patent No. 9,921,154 ('154 Patent) | "MULTIPLEXED DIGITAL ASSAYS" |

15. The Infringement Letter also included detailed "exemplary infringement claim charts" purporting to demonstrate how ChromaCode's HDPCR Assays allegedly infringe claim 1 of the '128 Patent and claim 1 of the '154 Patent. *See* Exhibit C, App'x 1 & 2.

16. The Infringement Letter further stated that Bio-Rad is open to "licensing discussions" regarding the Challenged Patents.

17. Neither ChromaCode, nor any of its products, directly or indirectly infringe either of the Challenged Patents.

18. Neither ChromaCode, nor any of its products, perform each element of any claim of the Challenged Patents, nor does ChromaCode instruct third parties to perform each element of any claim of the Challenged Patents.

19. For example, ChromaCode's products do not "calculat[e] an average level of each target in the partitions based on the R signals, wherein the level calculated accounts for a coincidence of all possible combinations of the more than R targets in the same individual partitions," as required by claim 1 of the '128 Patent.

20. Similarly, ChromaCode's products do not "determine[e] a respective level of each of the R targets from the data," as required by claim 1 of the '154 Patent. Nor do ChromaCode's products require "a partition count for a partition population positive for two of the R targets," as required by the same claim.

21. These are non-exhaustive examples of why ChromaCode does not infringe the Challenged Patents.

22. As a result of the Infringement Letter, ChromaCode is under reasonable apprehension that Bio-Rad will pursue claims that ChromaCode infringes the Challenged

Patents. Bio-Rad's patent infringement threats act as a sword of Damocles over ChromaCode's business opportunities.

23. As a small private biotech company, ChromaCode relies on investments from venture partners and collaborations with larger corporations. As Bio-Rad well knows, when a behemoth like Bio-Rad sends a letter threatening patent infringement, that makes it more difficult for a company like ChromaCode to attract such investment or collaboration. Without ever having to sue, Bio-Rad's *threats* of patent infringement are designed, and are likely, to scare off potential investors, collaborators, and acquirors. In the past six years, Bio-Rad has instituted 10 patent infringement actions against competitors all over the country.[1] Any potential investor, collaborator, or acquiror will know that Bio-Rad's Infringement Letter raises a serious threat of future litigation, especially *if* said potential investor, collaborator, or acquiror chooses to help make ChromaCode a significant competitive threat to Bio-Rad's multi-billion-dollar bottom line. Although the threatened litigation is meritless, its mere threat is enough to reduce ChromaCode's attractiveness to these potential third parties. This cloud of potential future litigation on ChromaCode's freedom to operate poses a significant threat to ChromaCode. Accordingly, this action is necessary to resolve this immediate, real and justiciable controversy.

## FIRST CAUSE OF ACTION

## NON-INFRINGEMENT OF U.S. PATENT NO. 9,222,128

24. ChromaCode repeats and realleges each and every allegation set forth in the above paragraphs and incorporates them by reference herein.

[1] *See Bio-Rad Labs., Inc. v. GigaGen, Inc.*, 3:22-cv-07205-AMO (N.D. Cal. Nov. 16, 2022); *Bio-Rad Labs., Inc. v. 10X Genomics, Inc.*, No. 3:20-cv-03207-VC (N.D. Cal. May 11, 2020); *Bio-Rad Labs., Inc. v. Dropworks, Inc.*, No. 1:20-cv-00506-RGA (D. Del. Apr. 14, 2020); *Bio-Rad Labs., Inc. v. 10X Genomics, Inc.*, No. 1:19-cv-12533-WGY (D. Mass. Dec. 18, 2019); *Bio-Rad Labs., Inc. v. 10X Genomics, Inc.*, No. 1:19-cv-01699-RGA (D. Del. Sept. 11, 2019); *Bio-Rad Labs., Inc. v. Stilla Techs., Inc.*, No. 1:19-cv-11587-WGY (D. Mass. Jul. 22, 2019); *Bio-Rad Labs., Inc. v. 10X Genomics, Inc.*, No. 1:18-cv-01679-RGA (D. Del. Oct. 25, 2018); *Institut Pasteur v. Abbott Labs.*, No. 1:17-cv-07104 (N.D. Ill. Oct. 2, 2017); *Bio-Rad Labs., Inc. v. 10X Genomics, Inc.*, No. 3:17-cv-04339-VC (N.D. Cal. Jul. 31, 2017); *Bio-Rad Labs., Inc. v. Thermo Fisher Sci. Inc.*, 1:17-cv-00469-RGA (D. Del. Apr. 25, 2017).

25. In view of the facts as alleged above, there is an actual, substantial, immediate, and justiciable controversy between ChromaCode and Bio-Rad regarding whether ChromaCode infringes any claim of the '128 Patent.

26. According to the face of the '128 Patent, Bio-Rad is the assignee of the '128 Patent. On information and belief, Bio-Rad owns all rights, title, and interest in and under the '128 Patent.

27. Bio-Rad has alleged and continues to allege that products made, used, or sold by ChromaCode, or ChromaCode products that utilize certain methods of use, are covered by the claims of the '128 Patent, and that ChromaCode is infringing the '128 Patent. Bio-Rad may bring suit on this matter at any time. In the meantime, ChromaCode is harmed by these false allegations.

28. ChromaCode does not infringe, induce infringement, or contribute to the infringement of any claim of the '128 Patent, either literally or under the doctrine of equivalents. ChromaCode has not made, used, sold, offered for sale, or imported any products that infringe, directly or indirectly, any claim of the '128 Patent.

29. Therefore, a substantial controversy exists between ChromaCode and Bio-Rad, parties having adverse legal interests, of sufficient immediacy and reality to warrant the issuance of a declaratory judgment that ChromaCode has not infringed and does not infringe any claim of the '128 Patent.

30. A substantial, immediate, real, and justiciable controversy exists between ChromaCode and Bio-Rad as to whether ChromaCode's products infringe the '128 Patent. ChromaCode accordingly requests a judicial determination of its rights, duties, and obligations regarding the '128 Patent.

31. ChromaCode seeks a judgment declaring that ChromaCode does not directly or indirectly infringe any claim of the '128 Patent.

**SECOND CAUSE OF ACTION**

**NON-INFRINGEMENT OF U.S. PATENT NO. 9,921,154**

32. ChromaCode repeats and realleges each and every allegation set forth in the above paragraphs and incorporates them by reference herein.

33. In view of the facts as alleged above, there is an actual, substantial, immediate, and justiciable controversy between ChromaCode and Bio-Rad regarding whether ChromaCode infringes any claim of the '154 Patent.

34. According to the face of the '154 Patent, Bio-Rad is the assignee of the '154 Patent. On information and belief, Bio-Rad owns all rights, title, and interest in and under the '154 Patent.

35. Bio-Rad has alleged and continues to allege that products made, used, or sold by ChromaCode, or ChromaCode products that utilize certain methods of use, are covered by the claims of the '154 Patent, and that ChromaCode is infringing the '154 Patent. Bio-Rad may bring suit on this matter at any time. In the meantime, ChromaCode is harmed by these false allegations.

36. ChromaCode does not infringe, induce infringement, or contribute to the infringement of any claim of the '154 Patent, either literally or under the doctrine of equivalents. ChromaCode has not made, used, sold, offered for sale, or imported any products that infringe, directly or indirectly, any claim of the '154 Patent.

37. Therefore, a substantial controversy exists between ChromaCode and Bio-Rad, parties having adverse legal interests, of sufficient immediacy and reality to warrant the issuance of a declaratory judgment that ChromaCode has not infringed and does not infringe any claim of the '154 Patent.

38. An actual and justiciable controversy exists between ChromaCode and Bio-Rad as to whether ChromaCode's products infringe the '154 Patent. ChromaCode accordingly requests a judicial determination of its rights, duties, and obligations regarding the '154 Patent.

39. ChromaCode seeks a judgment declaring that ChromaCode does not directly or indirectly infringe any claim of the '154 Patent.

**PRAYER FOR RELIEF**

ChromaCode respectfully requests the following relief:

a. That the Court enter a judgment declaring that ChromaCode has not infringed and does not infringe any claim of the Challenged Patents;

b. That the Court enter a judgment in favor of ChromaCode and against Bio-Rad on ChromaCode's claims;

c. A judgment declaring that Bio-Rad, and each of its officers, employees, agents, alter egos, attorneys, and any persons in active concert or participation with them, be restrained and enjoined from further prosecuting or instituting any action against ChromaCode claiming that the Challenged Patents are infringed, or from representing that any of ChromaCode's products, directly or indirectly, infringe the Challenged Patents;

d. Finding that this is an exceptional case under 35 U.S.C. § 285;

e. Awarding ChromaCode its costs and attorneys' fees in connection with this action; and

f. Granting ChromaCode such further and additional relief as the Court deems just and proper.

**JURY DEMAND**

ChromaCode hereby demands a jury trial on all issues and claims so triable.

Dated: September 20, 2023

WILSON SONSINI GOODRICH & ROSATI, PC

*/s/ Amy H. Candido*
AMY H. CANDIDO

*Attorneys for Plaintiff ChromaCode, Inc.*

# EXHIBIT A

US009222128B2

(12) **United States Patent**
**Saxonov et al.**

(10) **Patent No.: US 9,222,128 B2**
(45) **Date of Patent: Dec. 29, 2015**

(54) **MULTIPLEXED DIGITAL ASSAYS WITH COMBINATORIAL USE OF SIGNALS**

(75) Inventors: **Serge Saxonov**, Oakland, CA (US); **Simant Dube**, Pleasanton, CA (US); **Benjamin J. Hindson**, Livermore, CA (US); **Adam M. McCoy**, Davis, CA (US)

(73) Assignee: **Bio-Rad Laboratories, Inc.**, Hercules, CA (US)

( * ) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 345 days.

(21) Appl. No.: **13/424,304**

(22) Filed: **Mar. 19, 2012**

(65) **Prior Publication Data**

US 2012/0329664 A1 Dec. 27, 2012

**Related U.S. Application Data**

(60) Provisional application No. 61/454,373, filed on Mar. 18, 2011.

(51) **Int. Cl.**

| | |
|---|---|
| ***G01N 33/48*** | (2006.01) |
| ***G01N 33/50*** | (2006.01) |
| ***G01N 31/00*** | (2006.01) |
| ***C12Q 1/68*** | (2006.01) |
| ***G06F 19/20*** | (2011.01) |
| ***G06F 19/12*** | (2011.01) |
| ***G06F 19/18*** | (2011.01) |
| *G06F 17/10* | (2006.01) |
| *G06F 17/12* | (2006.01) |
| *G06F 17/11* | (2006.01) |

(52) **U.S. Cl.**

CPC ..... ***C12Q 1/6851*** (2013.01); ***G06F 19/12*** (2013.01); ***G06F 19/18*** (2013.01); ***G06F 19/20*** (2013.01); *G06F 17/10* (2013.01); *G06F 17/11* (2013.01); *G06F 17/12* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

| | | | |
|---|---|---|---|
| 3,575,220 | A | 4/1971 | Davis et al. |
| 4,051,025 | A | 9/1977 | Ito |
| 4,201,691 | A | 5/1980 | Asher et al. |
| 4,283,262 | A | 8/1981 | Cormier et al. |
| 4,348,111 | A | 9/1982 | Goulas et al. |
| 4,636,075 | A | 1/1987 | Knollenberg |
| 4,948,961 | A | 8/1990 | Hillman et al. |
| 5,055,390 | A | 10/1991 | Weaver et al. |
| 5,176,203 | A | 1/1993 | Larzul |
| 5,225,332 | A | 7/1993 | Weaver et al. |
| 5,270,183 | A | 12/1993 | Corbett et al. |

(Continued)

FOREIGN PATENT DOCUMENTS

| | | |
|---|---|---|
| EP | 1 522 582 A2 | 4/2005 |
| EP | 1 522 582 B1 | 4/2007 |

(Continued)

OTHER PUBLICATIONS

Dube et al. Mathematical Analysis of copy number Variation in a DNA Sample Using Digital PCR on a Nanofluidic Device (2008) PLoS ONE 3(8): e2876. doi:10.1371/journal.pone.0002876.

(Continued)

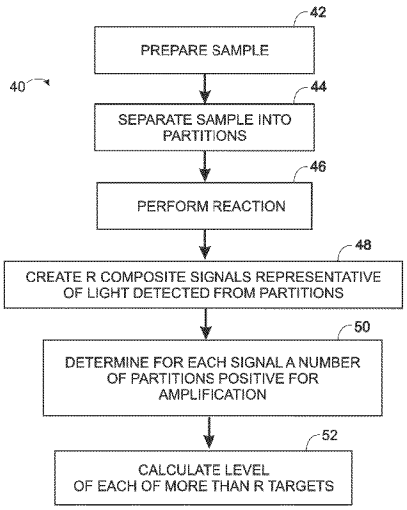
*Primary Examiner* — Russell S Negin

(74) *Attorney, Agent, or Firm* — Kolisch Hartwell, P.C.

(57) **ABSTRACT**

System, including methods, apparatus, and compositions, for performing a multiplexed digital assay on a greater number of targets through combinatorial use of signals.

**15 Claims, 10 Drawing Sheets**



(56) **References Cited**

U.S. PATENT DOCUMENTS

5,314,809 A 5/1994 Erlich et al.
5,344,930 A 9/1994 Riess et al.
5,422,277 A 6/1995 Connelly et al.
5,538,667 A 7/1996 Hill et al.
5,555,191 A 9/1996 Hripcsak
5,585,069 A 12/1996 Zanzucchi et al.
5,587,128 A 12/1996 Wilding et al.
5,602,756 A 2/1997 Atwood et al.
5,720,923 A 2/1998 Haff et al.
5,736,314 A 4/1998 Hayes et al.
5,779,977 A 7/1998 Haff et al.
5,827,480 A 10/1998 Haff et al.
5,856,174 A 1/1999 Lipshutz et al.
5,912,945 A 6/1999 Da Silva et al.
5,928,907 A 7/1999 Woudenberg et al.
5,945,334 A 8/1999 Besemer et al.
5,972,716 A 10/1999 Ragusa et al.
5,980,936 A 11/1999 Krafft et al.
5,994,056 A 11/1999 Higuchi
6,033,880 A 3/2000 Haff et al.
6,042,709 A 3/2000 Parce et al.
6,057,149 A 5/2000 Burns et al.
6,126,899 A 10/2000 Woudenberg et al.
6,130,098 A 10/2000 Handique et al.
6,143,496 A 11/2000 Brown et al.
6,146,103 A 11/2000 Lee et al.
6,171,785 B1 1/2001 Higuchi
6,175,669 B1 1/2001 Colston et al.
6,176,609 B1 1/2001 Cleveland et al.
6,177,479 B1 1/2001 Nakajima et al.
6,210,879 B1 4/2001 Meloni et al.
6,258,569 B1 7/2001 Livak et al.
6,281,254 B1 8/2001 Nakajima et al.
6,303,343 B1 10/2001 Kopf-Sill
6,357,907 B1 3/2002 Cleveland et al.
6,384,915 B1 5/2002 Everett et al.
6,391,559 B1 5/2002 Brown et al.
6,440,706 B1 8/2002 Vogelstein et al.
6,466,713 B2 10/2002 Everett et al.
6,488,895 B1 12/2002 Kennedy
6,489,103 B1 12/2002 Griffiths et al.
6,494,104 B2 12/2002 Kawakita et al.
6,509,085 B1 1/2003 Kennedy
6,521,427 B1 2/2003 Evans
6,524,456 B1 2/2003 Ramsey et al.
6,540,895 B1 4/2003 Spence et al.
6,551,841 B1 4/2003 Wilding et al.
6,558,916 B2 5/2003 Veerapandian et al.
6,575,188 B2 6/2003 Parunak
6,602,472 B1 8/2003 Zimmermann et al.
6,620,625 B2 9/2003 Wolk et al.
6,637,463 B1 10/2003 Lei et al.
6,638,749 B1 10/2003 Beckman et al.
6,660,367 B1 12/2003 Yang et al.
6,663,619 B2 12/2003 Odrich et al.
6,664,044 B1 12/2003 Sato
6,670,153 B2 12/2003 Stern
6,753,147 B2 6/2004 Vogelstein et al.
6,767,706 B2 7/2004 Quake et al.
6,773,566 B2 8/2004 Shenderov
6,808,882 B2 10/2004 Griffiths et al.
6,814,934 B1 11/2004 Higuchi
6,833,242 B2 12/2004 Quake et al.
6,900,021 B1 5/2005 Harrison et al.
6,905,885 B2 6/2005 Colston et al.
6,949,176 B2 9/2005 Vacca et al.
6,960,437 B2 11/2005 Enzelberger et al.
6,964,846 B1 11/2005 Shuber
7,010,391 B2 3/2006 Handique et al.
7,041,481 B2 5/2006 Anderson et al.
7,052,244 B2 5/2006 Fouillet et al.
7,081,336 B2 7/2006 Bao et al.
7,091,048 B2 8/2006 Parce et al.
7,094,379 B2 8/2006 Fouillet et al.
7,118,910 B2 10/2006 Unger et al.
7,129,091 B2 10/2006 Ismagilov et al.
7,138,233 B2 11/2006 Griffiths et al.
7,141,537 B2 11/2006 Audenaert et al.
7,192,557 B2 3/2007 Wu et al.
7,198,897 B2 4/2007 Wangh et al.
7,238,268 B2 7/2007 Ramsey et al.
7,244,567 B2 7/2007 Chen et al.
7,252,943 B2 8/2007 Griffiths et al.
7,268,167 B2 9/2007 Higuchi et al.
7,268,179 B2 9/2007 Brown
7,270,786 B2 9/2007 Parunak et al.
7,279,146 B2 10/2007 Nassef et al.
7,294,468 B2 11/2007 Bell et al.
7,294,503 B2 11/2007 Quake et al.
7,306,929 B2 12/2007 Ignatov et al.
7,312,085 B2 12/2007 Chou et al.
7,323,305 B2 1/2008 Leamon et al.
7,368,233 B2 5/2008 Shuber et al.
7,375,140 B2 5/2008 Higuchi et al.
7,423,751 B2 9/2008 Hairston et al.
7,429,467 B2 9/2008 Holliger et al.
7,567,596 B2 7/2009 Dantus et al.
7,579,172 B2 8/2009 Cho et al.
7,595,195 B2 9/2009 Lee et al.
7,622,280 B2 11/2009 Holliger et al.
7,629,123 B2 12/2009 Millonig et al.
7,776,927 B2 8/2010 Chu et al.
7,807,920 B2 10/2010 Linke et al.
7,842,457 B2 11/2010 Berka et al.
8,399,198 B2 3/2013 Hiddessen et al.
2001/0046701 A1 11/2001 Schulte et al.
2002/0021866 A1 2/2002 Everett et al.
2002/0022261 A1 2/2002 Anderson et al.
2002/0060156 A1 5/2002 Mathies et al.
2002/0068357 A1 6/2002 Mathies et al.
2002/0093655 A1 7/2002 Everett et al.
2002/0141903 A1 10/2002 Parunak et al.
2002/0142483 A1 10/2002 Yao et al.
2002/0151040 A1 10/2002 O'Keefe et al.
2002/0164820 A1 11/2002 Brown
2002/0195586 A1 12/2002 Auslander et al.
2003/0001121 A1 1/2003 Hochstein
2003/0003054 A1 1/2003 McDonald et al.
2003/0003441 A1 1/2003 Colston et al.
2003/0008308 A1 1/2003 Enzelberger et al.
2003/0027150 A1 2/2003 Katz
2003/0027244 A1 2/2003 Colston et al.
2003/0027352 A1 2/2003 Hooper et al.
2003/0032172 A1 2/2003 Colston, Jr. et al.
2003/0049659 A1 3/2003 Lapidus et al.
2003/0087300 A1 5/2003 Knapp et al.
2003/0170698 A1 9/2003 Gascoyne et al.
2003/0180765 A1 9/2003 Traverso et al.
2003/0204130 A1 10/2003 Colston, Jr. et al.
2004/0007463 A1 1/2004 Ramsey et al.
2004/0038385 A1 2/2004 Langlois et al.
2004/0067493 A1 4/2004 Matsuzaki et al.
2004/0068019 A1 4/2004 Higuchi et al.
2004/0074849 A1 4/2004 Brown et al.
2004/0171055 A1 9/2004 Brown
2004/0180346 A1 9/2004 Anderson et al.
2004/0208792 A1 10/2004 Linton et al.
2005/0036920 A1 2/2005 Gilbert
2005/0042639 A1 2/2005 Knapp et al.
2005/0064460 A1 3/2005 Holliger et al.
2005/0079510 A1 4/2005 Berka et al.
2005/0112541 A1 5/2005 Durack et al.
2005/0172476 A1 8/2005 Stone et al.
2005/0202429 A1 9/2005 Trau et al.
2005/0221279 A1 10/2005 Carter et al.
2005/0221373 A1 10/2005 Enzelberger et al.
2005/0227264 A1 10/2005 Nobile et al.
2005/0239192 A1 10/2005 Nasarabadi et al.
2005/0277125 A1 12/2005 Benn et al.
2005/0282206 A1 12/2005 Michael Corbett et al.
2006/0014187 A1 1/2006 Li et al.
2006/0057599 A1 3/2006 Dzenitis et al.
2006/0077755 A1 4/2006 Higuchi et al.
2006/0079583 A1 4/2006 Higuchi et al.

(56) **References Cited**

U.S. PATENT DOCUMENTS

2006/0079584 A1 4/2006 Higuchi et al.
2006/0079585 A1 4/2006 Higuchi et al.
2006/0094108 A1 5/2006 Yoder et al.
2006/0106208 A1 5/2006 Nochumson et al.
2006/0188463 A1 8/2006 Kim et al.
2007/0003442 A1 1/2007 Link et al.
2007/0010974 A1 1/2007 Nicoli et al.
2007/0048756 A1 3/2007 Mei et al.
2007/0109542 A1 5/2007 Tracy et al.
2007/0166200 A1 7/2007 Zhou et al.
2007/0195127 A1 8/2007 Ahn et al.
2007/0196397 A1 8/2007 Torii et al.
2007/0202525 A1 8/2007 Quake et al.
2007/0231393 A1 10/2007 Ritter et al.
2007/0242111 A1 10/2007 Pamula et al.
2007/0248956 A1 10/2007 Buxbaum et al.
2007/0258083 A1 11/2007 Heppell et al.
2007/0275415 A1 11/2007 Srinivasan et al.
2008/0003142 A1 1/2008 Link et al.
2008/0014589 A1 1/2008 Link et al.
2008/0038810 A1 2/2008 Pollack et al.
2008/0070862 A1 3/2008 Laster et al.
2008/0090244 A1 4/2008 Knapp et al.
2008/0138815 A1 6/2008 Brown et al.
2008/0145923 A1 6/2008 Hahn et al.
2008/0153091 A1 6/2008 Brown et al.
2008/0160525 A1 7/2008 Brown et al.
2008/0161420 A1 7/2008 Shuber
2008/0166793 A1 7/2008 Beer et al.
2008/0169184 A1 7/2008 Brown et al.
2008/0169195 A1 7/2008 Jones et al.
2008/0171324 A1 7/2008 Brown et al.
2008/0171325 A1 7/2008 Brown et al.
2008/0171326 A1 7/2008 Brown et al.
2008/0171327 A1 7/2008 Brown et al.
2008/0171380 A1 7/2008 Brown et al.
2008/0171382 A1 7/2008 Brown et al.
2008/0213766 A1 9/2008 Brown et al.
2008/0214407 A1 9/2008 Remacle et al.
2008/0262384 A1 10/2008 Wiederkehr et al.
2008/0268436 A1 10/2008 Duan et al.
2008/0274455 A1 11/2008 Puskas et al.
2008/0280331 A1 11/2008 Davies et al.
2008/0280865 A1 11/2008 Tobita
2008/0280955 A1 11/2008 McCamish
2008/0314761 A1 12/2008 Herminghaus et al.
2009/0012187 A1 1/2009 Chu et al.
2009/0026082 A1 1/2009 Rothberg et al.
2009/0029867 A1 1/2009 Reed et al.
2009/0035770 A1 2/2009 Mathies et al.
2009/0035838 A1 2/2009 Quake et al.
2009/0061428 A1 3/2009 McBride et al.
2009/0068170 A1 3/2009 Weitz et al.
2009/0069194 A1 3/2009 Ramakrishnan
2009/0098044 A1 4/2009 Kong et al.
2009/0114043 A1 5/2009 Cox
2009/0131543 A1 5/2009 Weitz et al.
2009/0162929 A1 6/2009 Ikeda
2009/0176271 A1 7/2009 Durack et al.
2009/0203063 A1 8/2009 Wheeler et al.
2009/0217742 A1 9/2009 Chiu et al.
2009/0220434 A1 9/2009 Sharma
2009/0235990 A1 9/2009 Beer
2009/0239308 A1 9/2009 Dube et al.
2009/0291435 A1 11/2009 Unger et al.
2009/0311713 A1 12/2009 Pollack et al.
2009/0325184 A1 12/2009 Woudenberg et al.
2009/0325234 A1 12/2009 Gregg et al.
2009/0325236 A1 12/2009 Griffiths et al.
2010/0009360 A1 1/2010 Rosell Costa et al.
2010/0020565 A1 1/2010 Seward
2010/0022414 A1 1/2010 Link et al.
2010/0041046 A1 2/2010 Chiu et al.
2010/0047808 A1 2/2010 Reed et al.
2010/0069250 A1 3/2010 White, III et al.
2010/0069263 A1 3/2010 Shendure et al.
2010/0092973 A1 4/2010 Davies et al.
2010/0137163 A1 6/2010 Link et al.
2010/0233686 A1 9/2010 Higuchi et al.
2010/0248385 A1 9/2010 Tan et al.
2010/0261229 A1 10/2010 Lau et al.
2010/0304446 A1 12/2010 Davies et al.
2010/0304978 A1 12/2010 Deng et al.
2011/0000560 A1 1/2011 Miller et al.
2011/0027394 A1 2/2011 McClements et al.
2011/0053798 A1 3/2011 Hindson et al.
2011/0070589 A1 3/2011 Belgrader et al.
2011/0086780 A1 4/2011 Colston, Jr. et al.
2011/0092373 A1 4/2011 Colston, Jr. et al.
2011/0092376 A1 4/2011 Colston, Jr. et al.
2011/0092392 A1 4/2011 Colston, Jr. et al.
2011/0118151 A1 5/2011 Eshoo et al.
2011/0160078 A1 6/2011 Fodor et al.
2011/0177563 A1 7/2011 Hahn et al.
2011/0183330 A1 7/2011 Lo et al.
2011/0212516 A1 9/2011 Ness et al.
2011/0217712 A1 9/2011 Hiddessen et al.
2011/0217736 A1 9/2011 Hindson
2011/0218123 A1 9/2011 Weitz et al.
2011/0244455 A1 10/2011 Larson et al.
2011/0250597 A1 10/2011 Larson et al.
2011/0311978 A1 12/2011 Makarewicz, Jr. et al.
2012/0021423 A1 1/2012 Colston, Jr. et al.
2012/0028311 A1 2/2012 Colston, Jr. et al.
2012/0122714 A1 5/2012 Samuels et al.
2012/0152369 A1 6/2012 Hiddessen et al.
2012/0171683 A1 7/2012 Ness et al.
2012/0190032 A1 7/2012 Ness et al.
2012/0190033 A1 7/2012 Ness et al.
2012/0194805 A1 8/2012 Ness et al.
2012/0208241 A1 8/2012 Link
2012/0219947 A1 8/2012 Yurkovetsky et al.
2012/0220494 A1 8/2012 Samuels et al.
2012/0264646 A1 10/2012 Link et al.
2012/0302448 A1 11/2012 Hutchison et al.
2012/0309002 A1 12/2012 Link
2013/0017551 A1 1/2013 Dube
2013/0040841 A1 2/2013 Saxonov et al.
2013/0045875 A1 2/2013 Saxonov et al.
2013/0059754 A1 3/2013 Tzonev
2013/0064776 A1 3/2013 El Harrak et al.
2013/0084572 A1 4/2013 Hindson et al.
2013/0099018 A1 4/2013 Miller et al.
2013/0109575 A1 5/2013 Kleinschmidt et al.

FOREIGN PATENT DOCUMENTS

GB 1 503 163 3/1978
GB 2 097 692 11/1982
JP 0295433 4/1990
WO 82/02562 8/1982
WO 84/02000 5/1984
WO 92/01812 2/1992
WO 94/05414 3/1994
WO 96/12194 4/1996
WO 98/00231 1/1998
WO 98/16313 4/1998
WO 98/44151 10/1998
WO 98/44152 10/1998
WO 98/47003 10/1998
WO 01/07159 2/2001
WO 01/12327 2/2001
WO 02/23163 3/2002
WO 02/060584 8/2002
WO 02/068104 9/2002
WO 02/081490 10/2002
WO 02/081729 10/2002
WO 03/016558 2/2003
WO 03/042410 5/2003
WO WO 03/064691 A2 * 8/2003
WO 03/072258 9/2003
WO 2004/040001 5/2004
WO 2005/007812 1/2005
WO 2005/010145 2/2005

(56) **References Cited**

FOREIGN PATENT DOCUMENTS

| | | | |
|---|---|---|---|
| WO | 2005/021151 | | 3/2005 |
| WO | 2005/023091 | | 3/2005 |
| WO | 2005/055807 | | 6/2005 |
| WO | 2005/073410 | | 8/2005 |
| WO | 2005/075683 | | 8/2005 |
| WO | WO 2006/002167 | A2 * | 1/2006 |
| WO | 2006/023719 | | 3/2006 |
| WO | 2006/027757 | | 3/2006 |
| WO | 2006/038035 | | 4/2006 |
| WO | 2006/086777 | | 8/2006 |
| WO | 2006/095981 | | 9/2006 |
| WO | 2007/091228 | | 8/2007 |
| WO | 2007/091230 | | 8/2007 |
| WO | 2007/092473 | | 8/2007 |
| WO | 2007/133710 | | 11/2007 |
| WO | 2008/021123 | | 2/2008 |
| WO | 2008/024114 | | 2/2008 |
| WO | 2008/063227 | | 5/2008 |
| WO | 2008/070074 | | 6/2008 |
| WO | 2008/070862 | | 6/2008 |
| WO | 2008/109176 | | 9/2008 |
| WO | 2008/109878 | | 9/2008 |
| WO | 2008/112177 | | 9/2008 |
| WO | 2009/002920 | | 12/2008 |
| WO | 2009/015863 | | 2/2009 |
| WO | 2009/049889 | | 4/2009 |
| WO | 2009/085246 | | 7/2009 |
| WO | 2010/001419 | | 1/2010 |
| WO | 2010/018465 | | 2/2010 |
| WO | 2010036352 | A1 | 4/2010 |
| WO | 2011/034621 | | 3/2011 |
| WO | 2011/079176 | | 6/2011 |

OTHER PUBLICATIONS

Young, Lee W., Authorized officer, International Searching Authority, International Search Report, PCT Patent Application Serial No. PCT/US2012/029712; search date: Aug. 7, 2012; mail date: Aug. 17, 2012.

Young, Lee W., Authorized officer, International Searching Authority, Written Opinion of the International Searching Authority, PCT Patent Application Serial No. PCT/US2012/029712; opinion date: Aug. 7, 2012; mail date: Aug. 17, 2012.

Zhong et al. Multiplex digital PCR: breaking the one target per color barrier of quantitative PCR. Lab Chip, 2011, 11, 2167-2174, published May 17, 2011.

J. Smid-Korbar et al., "Efficiency and usability of silicone surfactants in emulsions," International Journal of Cosmetic Science 12, pp. 135-139, (1990), presented at the 15th IFSCC International Congress, Sep. 26-29, 1988, London.

A. Chittofrati et al., "Perfluoropolyether microemulsions," Progress in Colloid & Polymer Science 79, pp. 218-225, (1989).

Steven A. Snow, "Synthesis and Characterization of Zwitterionic Silicone Sulfobetaine Surfactants," Langmuir, vol. 6, No. 2, American Chemical Society, pp. 385-391, (1990).

Polydimethylsiloxane, 5 pgs., published in FNP 52 (1992).

Russell Higuchi et al., "Kinetic PCR Analysis: Real-time Monitoring of DNA Amplification Reactions," Bio/Technology vol. II, pp. 1026-1030, Sep. 11, 1993.

D. A. Newman et al., "Phase Behavior of Fluoroether-Functional Amphiphiles in Supercritical Carbon Dioxide," The Journal of Supercritical Fluids, vol. 6, No. 4, pp. 205-210, (1993).

Y. Sela et al., "Newly designed polysiloxane-graft-poly (oxyethylene) copolymeric surfactants: preparation, surface activity and emulsification properties," Colloid & Polymer Science 272, pp. 684-691, (1994).

M. Gasperlin et al., "The structure elucidation of semisolid w/o emulsion systems containing silicone surfactant," International Journal of Pharmaceutics 107, pp. 51-56, (1994).

Mieczyslaw A. Piatyszek et al., "Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP)," Methods in Cell Science 17, pp. 1-15, (1995).

Anthony P. Shuber et al., "A Simplified Procedure for Developing Multiplex PCRs," Genome Research, published by Cold Spring Harbor Laboratory Press, pp. 488-493, (1995).

A. V. Yazdi et al., "Highly Carbon Dioxide Soluble Surfactants, Dispersants and Chelating Agents," Fluid Phase Equilibria, vol. 117, pp. 297-303, (1996).

Ariel A. Avilion et al., "Human Telomerase RNA and Telomerase Activity in Immortal Cell Lines and Tumor Tissues," Cancer Research 56, pp. 645-650, Feb. 1, 1996.

Shuming Nie et al., "Optical Detection of Single Molecules," Annu. Rev. Biophys. BiomoL Struct. vol. 26, pp. 567-596, (1997).

Edith J. Singley et al., "Phase behavior and emulsion formation of novel fluoroether amphiphiles in carbon dioxide," Fluid Phase Equilibria 128, pp. 199-219, (1997).

Olga Kalinina et al., "Nanoliter scale PCR with TaqMan Detection," Nucleic Acids Research, vol. 25, No. 10 pp. 1999-2004, (1997).

Zhen Guo et al., "Enhanced discrimination of single nucleotide polymorphisms by artificial mismatch hybridization," Nature Biotechnology vol. 15, pp. 331-335, Apr. 1997.

E. G. Ghenciu et al., "Affinity Extraction into Carbon Dioxide. 1. Extraction of Avidin Using a Biotin-Functional Fluoroether Surfactant," Ind. Eng. Chem. Res. vol. 36, No. 12, pp. 5366-5370, Dec. 1, 1997.

Paschalis Alexandridis, Structural Polymorphism of Poly(ethylene oxide)-Poly(propylene oxide) Block Copolymers in Nonaqueous Polar Solvents, Macromolecules, vol. 31, No. 20, pp. 6935-6942, Sep. 12, 1998.

Sandro R. P. Da Rocha et al., "Effect of Surfactants on the Interfacial Tension and Emulsion Formation between Water and Carbon Dioxide," Langmuir, vol. 15, No. 2, pp. 419-428, (1999), published on web Dec. 29, 1998.

Bert Vogelstein et al., "Digital PCR," Proc. Natl. Acad. Sci. USA, vol. 96, pp. 9236-9241, Aug. 1999.

Anthony J. O'Lenick, Jr., "Silicone Emulsions and Surfactants," Journal of Surfactants and Detergents, vol. 3, No. 3, Jul. 2000.

N. Garti et al., "Water Solubilization in Nonionic Microemulsions Stabilized by Grafted Siliconic Emulsifiers," Journal of Colloid and Interface Science vol. 233, pp. 286-294, (2001).

Shinji Katsura et al., "Indirect micromanipulation of single molecules in water-in-oil emulsion," Electrophoresis, vol. 22, pp. 289-293, (2001).

Hironobu Kunieda et al., "Effect of Hydrophilic- and Hydrophobic-Chain Lengths on the Phase Behavior of A-B-type Silicone Surfactants in Water," J. Phys. Chem. B, vol. 105, No. 23, pp. 5419-5426, (2001).

Hidenori Nagai et al., "Development of a Microchamber Array for Picoliter PCR," Analytical Chemistry, vol. 73, No. 5, pp. 1043-1047, Mar. 1, 2001.

Christopher B. Price, "Regular Review Point of Care Testing," BMJ, vol. 322, May 26, 2001.

3M Specialty Materials, "3M Fluorinert Electronic Liquid FC-3283," product information guide, issued Aug. 2001.

Ivonne Schneegaß et al., "Miniaturized flow-through PCR with different template types in a silicon chip thermocycler," Lab on a Chip, vol. 1, pp. 42-49, (2001).

Randla M. Hill, "Silicone surfactants—new developments," Current Opinion in Colloid & Interface Science 7, pp. 255-261, (2002).

Richard M. Cawthon, "Telomere measurement by quantitative PCR," Nucleic Acids Research, vol. 30, No. 10, pp. 1-6, (2002).

Anfeng Wang et al., "Direct Force Measurement of Silicone- and Hydrocarbon-Based ABA Triblock Surfactants in Alcoholic Media by Atomic Force Mircroscopy," Journal of Colloid and Interface Science 256, pp. 331-340 (2002).

Shelley L. Anna et al., "Formation of dispersions using "flow focusing" in microchannels," Applied Physics Letters, vol. 82, No. 3, Jan. 20, 2003.

Goldschmidt GMBH, "Abil® EM 90 Emulsifier for the formulation of cosmetic W/O creams and lotions," degussa. creating essentials brochure, pp. 1-7, May 2003.

Purnendu K. Dasgupta et al., "Light emitting diode-based detectors Absorbance, fluorescence and spectroelectrochemical measurements in a planar flow-through cell," Analytica Chimica Acta 500, pp. 337-364, (2003).

(56) **References Cited**

OTHER PUBLICATIONS

R. G. Rutledge et al., "Mathematics of quantitative kinetic PCR and the application of standard curves," Nucleic Acids Research, vol. 31, No. 16, pp. 1-6, (2003).
Chunming Ding et al., "Direct molecular haplotyping of long-range genomic DNA with M1-PCR," PNAS, vol. 100, No. 13, pp. 7449-7453, Jun. 24, 2003.
Devin Dressman et al., "Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations," PNAS, vol. 100, No. 15, Jul. 22, 2003.
Ulf Landegren et al., "Padlock and proximity probes for in situ and array-based analyses: tools for the post-genomic era," Comp. Funct. Genom, vol. 4, pp. 525-530, (2003).
Gudrun Pohl et al., "Principle and applications of digital PCR" review, www.future-drugs.com, Expert Rev. Mol. Diagn. 4(1), pp. 41-47, (2004).
Groff M. Schroeder et al., "Introduction to Flow Cytometry" version 5.1, 182 pgs. (2004).
Stéphane Swillens et al., "Instant evaluation of the absolute initial number of cDNA copies from a single real-time PCR curve," Nucleic Acids Research, vol. 32, No. 6, pp. 1-6, (2004).
Mats Gullberg et al., "Cytokine detection by antibody-based proximity ligation," PNAS, vol. 101, No. 22, pp. 8420-8424, Jun. 1, 2004.
Tianhao Zhang et al., "Behavioral Modeling and Performance Evaluation of Microelectrofluidics-Based PCR Systems Using SystemC," IEEE Transactions on Computer-Aided Design of Integrated Circuits and Systems, vol. 23, No. 6, pp. 843-858, Jun. 2004.
R. G. Rutledge, "Sigmoidal curve-fitting redefines quantitative real-time PCR with the prospective of developing automated high-throughput applications," Nucleic Acids Research. vol. 32, No. 22, pp. 1-8, (2004).
L. Spencer Roach et al., "Controlling Nonspecific Protein Absorption in a Plug-Based Microfluidic System by Controlling Interfacial Chemistry Using Fluorous-Phase Surfactants," Analytical Chemistry vol. 77, No. 3, pp. 785-796, Feb. 1, 2005.
Kevin D. Dorfman et al., "Contamination-Free Continuous Flow Microfluidic Polymerase Chain Reaction for Quantitative and Clinical Applications," Analytical Chemistry vol. 77, No. 11, pp. 3700-3704, Jun. 1, 2005.
James G. Wetmur et al., "Molecular haplotyping by linking emulsion PCR: analysis of paraoxonase 1 haplotypes and phenotypes," Nucleic Acids Research, vol. 33, No. 8, pp. 2615-2619, (2005).
Piotr Garstecki et al., "Mechanism for Flow-Rate Controlled Breakup in Confined Geometries: a Route to Monodisperse Emulsions," Physical Review Letters, 164501, pp. 164501-1-164501-4, Apr. 29, 2005.
Anna Musyanovych et al., "Miniemulsion Droplets as Single Molecule Nanoreactors for Polymerase Chain Reaction," Biomacromolecules, vol. 6, No. 4, pp. 1824-1828, (2005).
Max Chabert et al., "Droplet fusion by alternating current (AC) field electrocoalescence in microchannels," Electrophoresis, vol. 26, pp. 3706-3715, (2005).
Takaaki Kojima et al., "PCR amplification from single DNA molecules on magnetic beads in emulsion: application for high-throughput screening of transcription factor targets," Nucleic Acids Research, vol. 33, No. 17, pp. 19, (2005).
Marcel Margulies et al., "Genome sequencing in microfabricated high-density picolitre reactors," Nature, vol. 437, 51 pgs., Sep. 15, 2005.
Kristofer J. Thurecht et al., "Investigation of spontaneous microemulsion formation in supercritical carbon dioxide using high-pressure NMR," Journal of Supercritical Fluids, vol. 38, pp. 111-118, (2006).
Toshko Zhelev et al., "Heat Integration in Micro-Fluidic Devices," 16$^{th}$ European Symposium on Computer Aided Process Engineering and 9$^{th}$ International Symposium on Process Systems Engineering, pp. 1863-1868 published by Elsevier B.V. (2006).
Piotr Garstecki et al., "Formation of droplets and bubbles in a microfluidic T-junction—scaling and mechanism of break-up," Lab on a Chip, vol. 6, pp. 437-446, (2006).
Darren R. Link et al., "Electric Control of Droplets in Microfluidic Devices," Angewandte Chemie Int. Ed., vol. 45, pp. 2556-2560, (2006).
Peter Fielden et al., "Micro-Droplet Technology for High Throughout Systems and Methods," 1 pg., Mar. 8, 2006.
David Emerson et al., "Microfluidic Modelling Activities at C3M," Centre for Microfluidics & Microsystems Modelling, Daresbury Laboratory, pp. 1-26, May 15, 2006.
Richard Williams et al., "Amplification of complex gene libraries by emulsion PCR," Nature Methods, vol. 3, No. 7, pp. 545-550, Jul. 2006.
John H. Leamon et al., "Overview: methods and applications for droplet compartmentalization of biology," Nature Methods, vol. 3, No. 7, pp. 541-543, Jul. 2006.
Andrew D. Griffiths et al., "Miniaturising the laboratory in emulsion droplets," Trends in Biotechnology, vol. 24, No. 9, pp. 395-402, Jul. 14, 2006.
Jian-Bing Fan et al., "Highly parallel genomic assays," Nature Reviews/Genetics, vol. 7, pp. 632-644, Aug. 2006.
Jonas Jarvius et al., "Digital quantification using amplified single-molecule detection," Nature Methods, vol. 3, No. 9, pp. 15 pgs, Sep. 2006.
Kan Liu et al., "Droplet-based synthetic method using microflow focusing and droplet fusion," Microfluid Nanfluid, vol. 3, pp. 239-243, (2007), published online Sep. 22, 2006.
Dimitris Glotsos et al., "Robust Estimation of Bioaffinity Assay Fluorescence Signals," IEEE Transactions on Information Technology in Biomedicine, vol. 10, No. 4, pp. 733-739, Oct. 2006.
Kristofer J. Thurecht et al., "Kinetics of Enzymatic Ring-Opening Polymerization of -Caprolactone in Supercritical Carbon Dioxide," Macromolecules, vol. 39, pp. 7967-7972, (2006).
Machiko Hori et al., "Uniform amplification of multiple DNAs by emulsion PCR," Biochemical and Biophysical Research Communications, vol. 352, pp. 323-328, (2007).
Frank Diehl et al., "Digital quantification of mutant DNA in cancer patients," Current Opinion in Oncology, vol. 19, pp. 36-42, (2007).
Delai L. Chen et al., "Using Three-Phase Flow of Immiscible Liquids to Prevent Coalescence of Droplets in Microfluidic Channels: Criteria to Identify the Third Liquid and Validation with Protein Crystallization," Langmuir, vol. 23, No. 4, pp. 2255-2260, (2007).
S. Mohr et al., "Numerical and experimental study of a droplet-based PCR chip," Microfluid Nanofluid, vol. 3, pp. 611-621, (2007).
Sigrun M. Gustafsdottir et al., "In vitro analysis of DNA-protein interactions by proximity ligation," PNAS, vol. 104, No. 9, pp. 3067-3072, Feb. 27, 2007.
Daniel J. Diekema et al., "Look before You Leap: Active Surveillance for Multidrug-Resistant Organisms," Healthcare Epidemiology • CID 2007:44, pp. 1101-1107 (Apr. 15), electronically published Mar. 2, 2007.
Charles N. Baroud et al., "Thermocapillary valve for droplet production and sorting," Physical Review E 75, 046302, pp. 046302-1-046302-5, Apr. 5, 2007.
Qinyu Ge et al., "Emulsion PCR-based method to detect Y chromosome microdeletions," Analytical Biochemistry, vol. 367, pp. 173-178, May 10, 2007.
Chunsun Zhang et al., "Miniaturized PCR chips for nucleic acid amplification and analysis: latest advances and future trends," Nucleic Acids Research, vol. 35, No. 13, pp. 4223-4237, Jun. 18, 2007.
Y. M. Dennis Lo et al., "Digital PCR for the molecular detection of fetal chromosomal aneuploidy," PNAS, vol. 104, No. 32, pp. 13116-13121, Aug. 7, 2007.
Dayong Jin et al., "Practical Time-Gated Luminescence Flow Cytometry. II: Experimental Evaluation Using UV LED Excitation," Cytometry Part A • 71A, pp. 797-808, Aug. 24, 2007.
Helen R. Hobbs et al., "Homogeneous Biocatalysis in both Fluorous Biphasic and Supercritical Carbon Dioxide Systems," Angewandte Chemie, vol. 119, pp. 8006-8009, Sep. 6, 2007.
Nathan Blow, "PCR's next frontier," Nature Methods, vol. 4, No. 10, pp. 869-875, Oct. 2007.
Nicole Pamme, "continuous flow separations in microfluidic devices," Lab on a Chip, vol. 7, pp. 1644-1659, Nov. 2, 2007.

(56) **References Cited**

OTHER PUBLICATIONS

N. Reginald Beer et al., "On-Chip, Real-Time, Single-Copy Polymerase Chain Reaction in Picoliter Droplets," Analytical Chemistry, vol. 79, No. 22, pp. 8471-8475, Nov. 15, 2007.
Yuejun Zhao et al., "Microparticle Concentration and Separation by Traveling-Wave Dielectrophoresis (twDEP) for Digital Microfluidics," Journal of Microelectromechanical Systems, vol. 16, No. 6, pp. 1472-1481, Dec. 2007.
SIGMA-ALDRICH, "Synthesis of Mesoporous Materials," Material Matters, 3.1, 17, (2008).
Nick J. Carroll et al., "Droplet-Based Microfluidics for Emulsion and Solvent Evaporation Synthesis of Monodisperse Mesoporous Silica Microspheres," Langmuir, vol. 24, No. 3, pp. 658-661, Jan. 3, 2008.
Shia-Yen Teh et al., "Droplet microfluidics," Lab on a Chip, vol. 8, pp. 198-220, Jan. 11, 2008.
Chloroform (Phenomenex), Solvent Miscibility Table, Internet Archive WayBackMachine, 3 pgs., Feb. 1, 2008.
N. Reginald Beer et al., "On-Chip Single-Copy Real-Time Reverse-Transcription PCR in Isolated Picoliter Droplets," Analytical Chemistry, vol. 80, No. 6, pp. 1854-1858, Mar. 15, 2008.
Palani Kumaresan et al., "High-Throughput Single Copy DNA Amplification and Cell Analysis in Engineered Nanoliter Droplets," Analytical Chemistry, 17 pgs., Apr. 15, 2008.
Somil C. Mehta et a., "Mechanism of Stabilization of Silicone Oil—Water Emulsions Using Hybrid Siloxane Polymers," Langmuir, vol. 24, No. 9, pp. 4558-4563, Mar. 26, 2008.
Rhutesh K. Shah et al., "Polymers fit for function Making emulsions drop by drop," Materials Today, vol. 11, No. 4, pp. 18-27, Apr. 2008.
Mohamed Abdelgawad et al., "All-terrain droplet actuation," Lab on a Chip, vol. 8, pp. 672-677, Apr. 2, 2008.
Lung-Hsin Hung et al., "Rapid microfabrication of solvent-resistant biocompatible microfluidic devices," Lab on a Chip, vol. 8, pp. 983-987, Apr. 8, 2008.
Jenifer Clausell-Tormos et al., "Droplet-Based Microfluidic Platforms for the Encapsulation and Screening of Mammalian Cells and Multicellular Organisms," Chemistry & Biology, vol. 15, pp. 427-437, May 2008.
Vivienne N. Luk et al., "Pluronic Additives: A Solution to Sticky Problems in Digital Microfluidics," Langmuir, vol. 24, No. 12, pp. 6382-6289, May 16, 2008.
Yen-Heng Lin et al., "Droplet Formation Utilizing Controllable Moving-Wall Structures for Double-Emulsion Applications," Journal of Microelectromechanical Systems, vol. 17, No. 3, pp. 573-581, Jun. 2008.
Jian Qin et al., "Studying copy number variations using a nanofluidic platform," Nucleic Acids Research, vol. 36, No. 18, pp. 1-8, Aug. 18, 2008.
C. Holtze et al., "Biocompatible surfactants for water-in-fluorocarbon emulsions," Lab on a Chip, vol. 8, pp. 1632-1639, Sep. 2, 2008.
Margaret Macris Kiss et al., "High-Throughput Quantitative Polymerase Chain Reaction in Picoliter Droplets," Analytical Chemistry, 8 pgs., downloaded Nov. 17, 2008.
Jay Shendure et al., "Next-generation DNA sequencing," Nature Biotechnology, vol. 26, No. 10, pp. 1135-1145, Oct. 2008.
Bernhard G. Zimmermann et al., "Digital PCR: a powerful new tool for noninvasive prenatal diagnosis?," Prenatal Diagnosis, vol. 28 pp. 1087-1093, Nov. 10, 2008.
Avishay Bransky et al., "A microfluidic droplet generator based on a piezoelectric actuator," Lab on a Chip, vol. 9, pp. 516-520, Nov. 20, 2008.
David A. Weitz, "Novel Surfactants for Stabilizing Emulsions of Water or Hydrocarbon Oil-Based Droplets in a Fluorocarbon Oil Continuous Phase," Harvard Office of Technology Development: Available Technologies, pp. 1-3, downloaded Nov. 28, 2008.
Neil Reginald Beer et al., "Monodisperse droplet generation and rapid trapping for single molecule detection and reaction kinetics measurement," Lab on a Chip, vol. 9, pp. 841-844, Dec. 5, 2008.
Richard M. Cawthon, "Telomere length measurement by a novel monochrome multiplex quantitative PCR method," Nucleic Acids Research, vol. 37, No. 3, pp. 1-7, (2009).
Anthony J. O'Lenick, Jr., "Silicone Emulsions and Surfactants—A Review," Silicone Spectator, Silitech LLC, May 2009 (original published May 2000).
Adam R. Abate et al., "Functionalized glass coating for PDMS microfluidic devices," Lab on a Chip Technology: Fabrication and Microfluidics, 11 pgs., (2009).
Chia-Hung Chen et al., "Janus Particles Templated from Double Emulsion Droplets Generated Using Microfluidics," Langmuir, vol. 29, No. 8, pp. 4320-4323, Mar. 18, 2009.
Luis M. Fidalgo et al., "Coupling Microdroplet Microreactors with Mass Spectrometry: Reading the Contents of Single Droplets Online," Angewandte Chemie, vol. 48, pp. 3665-3668, Apr. 7, 2009.
Linas Mazutis et al., "A fast and efficient microfluidic system for highly selective one-to-one droplet fusion," Lab on a Chip, vol. 9, pp. 2665-2672, Jun. 12, 2009.
Linas Mazutis et al., "Droplet-Based Microfluidic Systems for High-Throughput Single DNA Molecule Isothermal Amplification and Analysis," Analytical Chemistry, vol. 81, No. 12, pp. 4813-4821, Jun. 15, 2009.
Frank McCaughan et al., "Single-molecule genomics," Journal of Pathology, vol. 220, pp. 297-306, Nov. 19, 2009.
Suzanne Weaver et al., "Taking qPCR to a higher level: Analysis of CNV reveals the power of high throughput qPCR to enhance quantitative resolution," Methods, vol. 50, pp. 271-276, Jan. 15, 2010.
Amelia L. Markey et al., "High-throughput droplet PCR," Methods, vol. 50, pp. 277-281, Feb. 2, 2010.
Yoon Sung Nam et al., "Nanosized Emulsions Stabilized by Semisolid Polymer Interphase," Langmuir, ACS Publications, Jul. 23, 2010.
Tatjana Schütze et al., "A streamlined protocol for emulsion polymerase chain reaction and subsequent purification," Analytical Biochemistry, vol. 410, pp. 155-157, Nov. 25, 2010.
Somanath Bhat et al., "Effect of sustained elevated temperature prior to amplification on template copy number estimation using digital polymerase chain reaction," Analyst, vol. 136, pp. 724-732, (2011).
James G. Wetmur, et al., "Linking Emulsion PCR Haplotype Analysis," PCR Protocols, Methods in Molecular Biology, vol. 687, pp. 165-175, (2011).
Paul Vulto et al., "Phaseguides: a paradigm shift in microfluidic priming and emptying," Lab on a Chip, vol. 11, No. 9, pp. 1561-1700, May 7, 2011.
Jiaqi Huang et al., "Rapid Screening of Complex DNA Samples by Single-Molecule Amplification and Sequencing," PLoS ONE, vol. 6, Issue 5, pp. 1-4, May 2011.
Burcu Kekevi et al., Synthesis and Characterization of Silicone-Based Surfactants as Anti-Foaming Agents, J. Surfact Deterg (2012), vol. 15, pp. 73-81, published online Jul. 7, 2011.
Leonardo B. Pinheiro et al., "Evaluation of a Droplet Digital Polymerase Chain Reaction Format for DNA Copy Number Quantification," Analytical Chemistry, vol. 84, pp. 1003-1011, Nov. 28, 2011.
Nicole L. Solimini et al., "Recurrent Hemizygous Deletions in Cancers May Optimize Proliferative Potential," Science, vol. 337, pp. 104-109, Jul. 6, 2012.
Labsmith, "Microfluid Components" webpage, downloaded Jul. 11, 2012.
Labsmith, "CapTite™ Microfluidic Interconnects" webpage, downloaded Jul. 11, 2012.
Nathan A. Tanner et al., "Simultaneous multiple target detection in real-time loop-mediated isothermal amplification," BioTechniques, vol. 53, pp. 8-19, Aug. 2012.
Stavros Therianos et al., "Single-Channel Quantitative Multiplex Reverse Transcriptase-Polymerase Chain Reaction for Large Numbers of Gene Products Differentiates Nondemented from Neuropathological Alzheimer's Disease", American Journal of Pathology, vol. 164, No. 3, Mar. 2004, pp. 795-806.
Yolanda Schaerli et al., "The potential of microfluidic water-in-oil droplets in experimental biology", Molecular BioSystems, Royal Society of Chemistry, vol. 5, No. 12, Dec. 1, 2009, pp. 1392-1404.
Zhishan Hua et al., "Multiplexed Real-Time Polymerase Chain Reaction on a digital Microfluidic Platform", Analytical Chemistry, vol. 82, No. 6, Mar. 15, 2010, pp. 2310-2316.
European Patent Office, "Supplementary European Search Report" in connection with related European Patent Application No. 12760944.4, dated Jan. 15, 2015, 9 pages.

* cited by examiner

Fig. 1

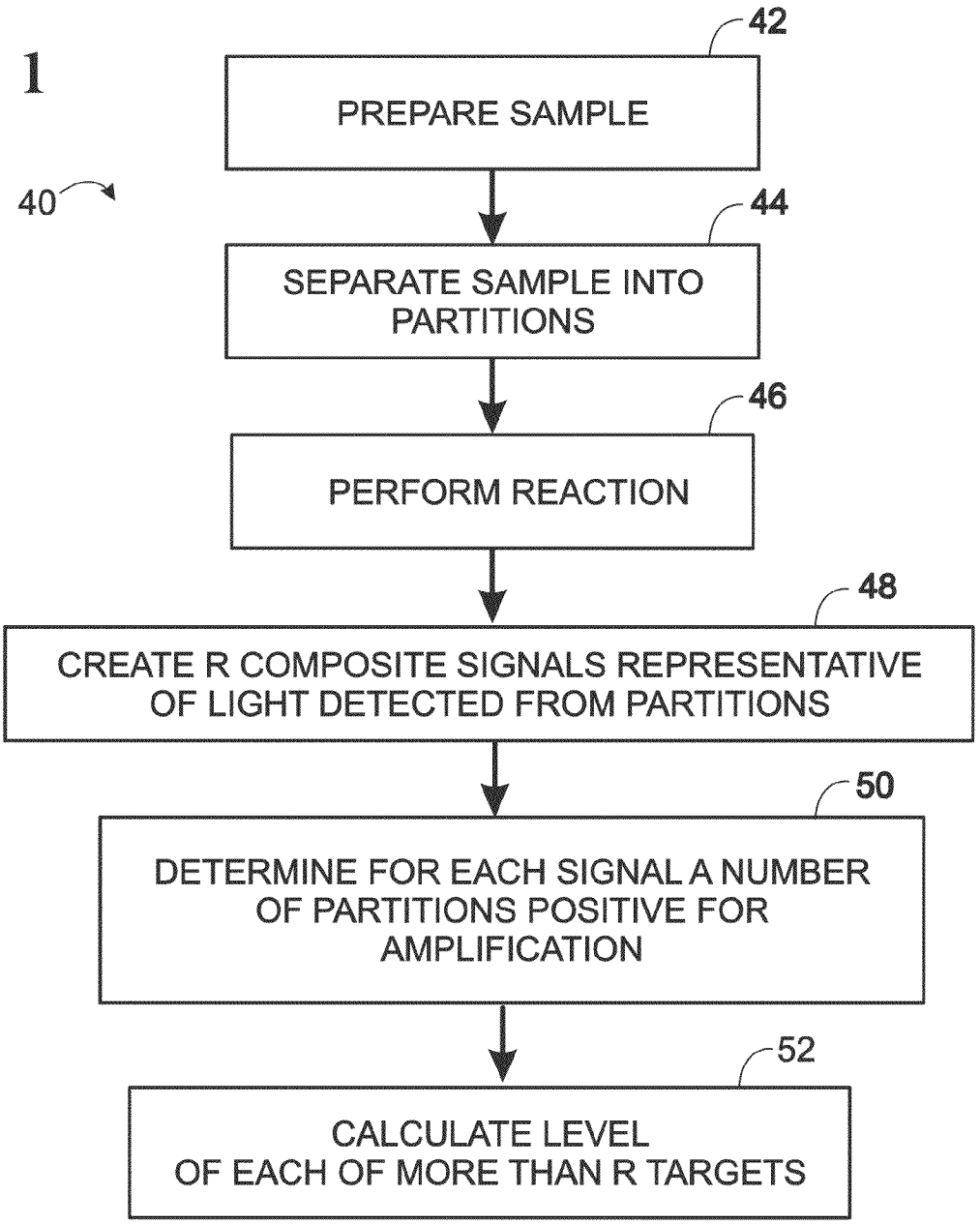

40
42
PREPARE SAMPLE
44
SEPARATE SAMPLE INTO PARTITIONS
46
PERFORM REACTION
48
CREATE R COMPOSITE SIGNALS REPRESENTATIVE OF LIGHT DETECTED FROM PARTITIONS
50
DETERMINE FOR EACH SIGNAL A NUMBER OF PARTITIONS POSITIVE FOR AMPLIFICATION
52
CALCULATE LEVEL OF EACH OF MORE THAN R TARGETS

Fig. 2

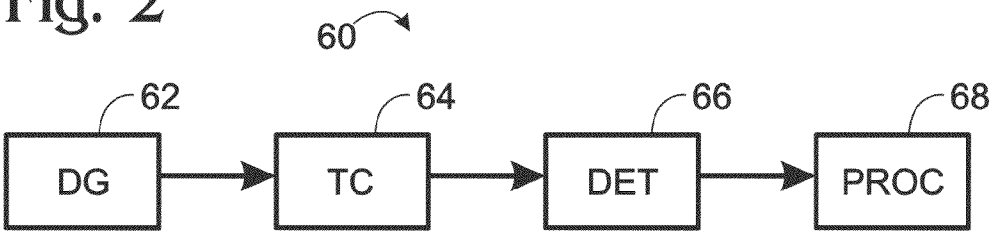

60
62
DG
64
TC
66
DET
68
PROC

Fig. 3

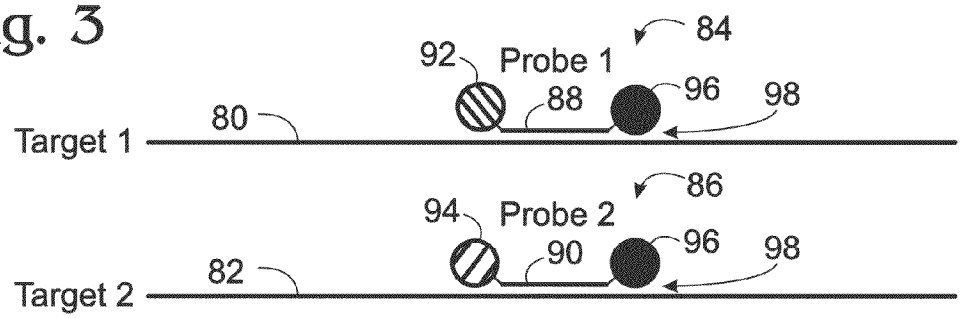



Fig. 4

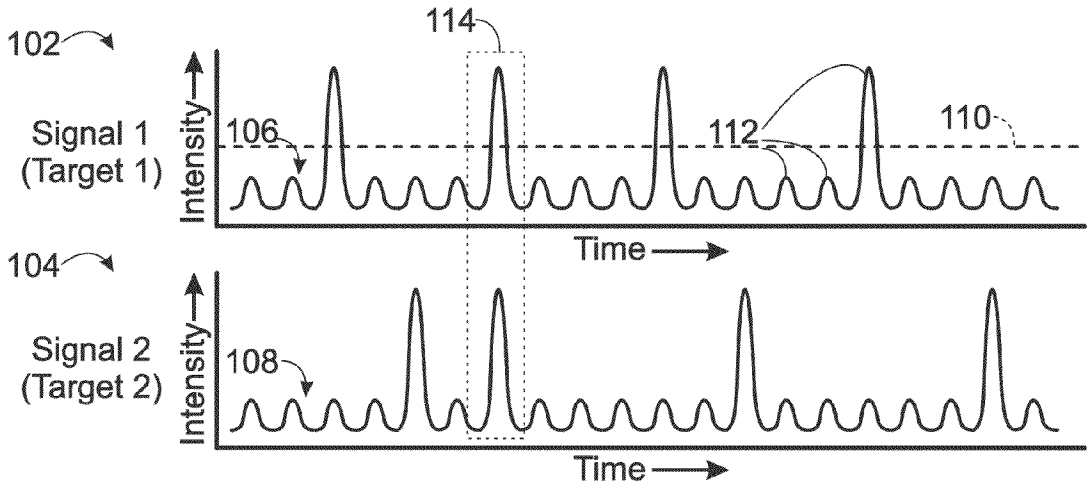



Fig. 5

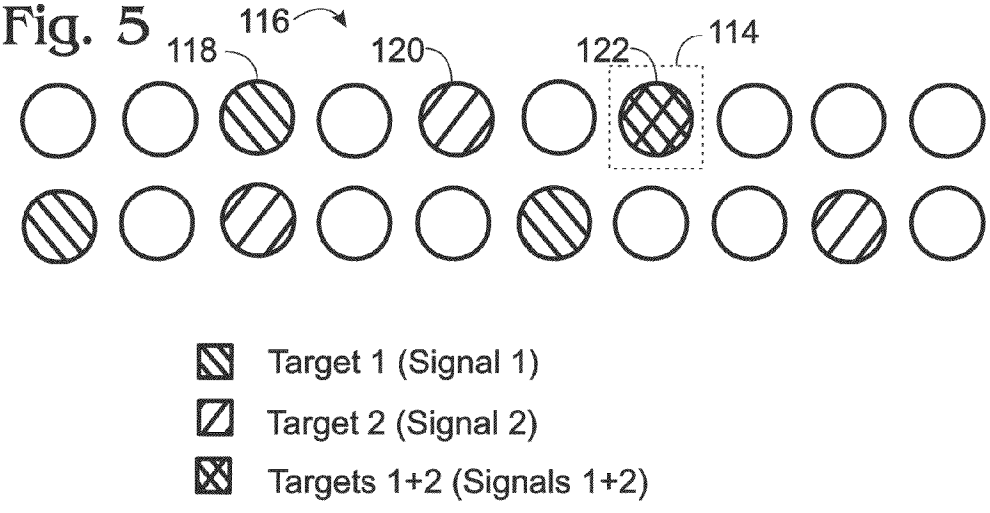


Fig. 6

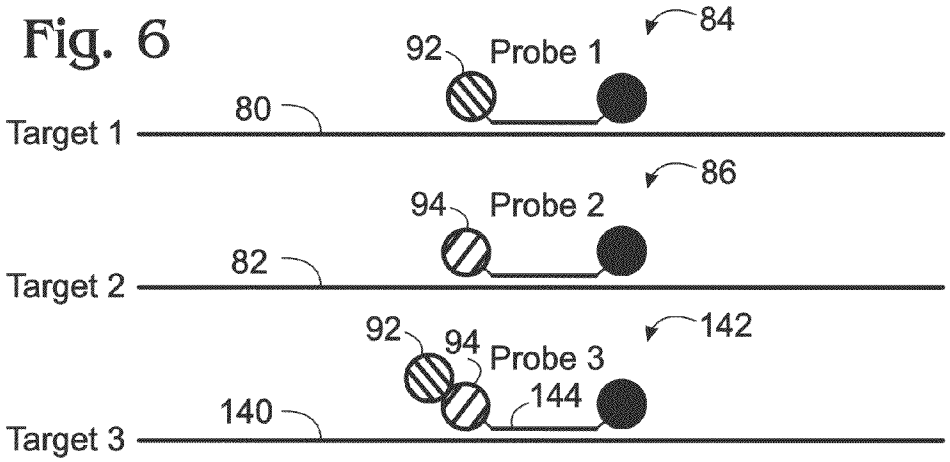



Fig. 7

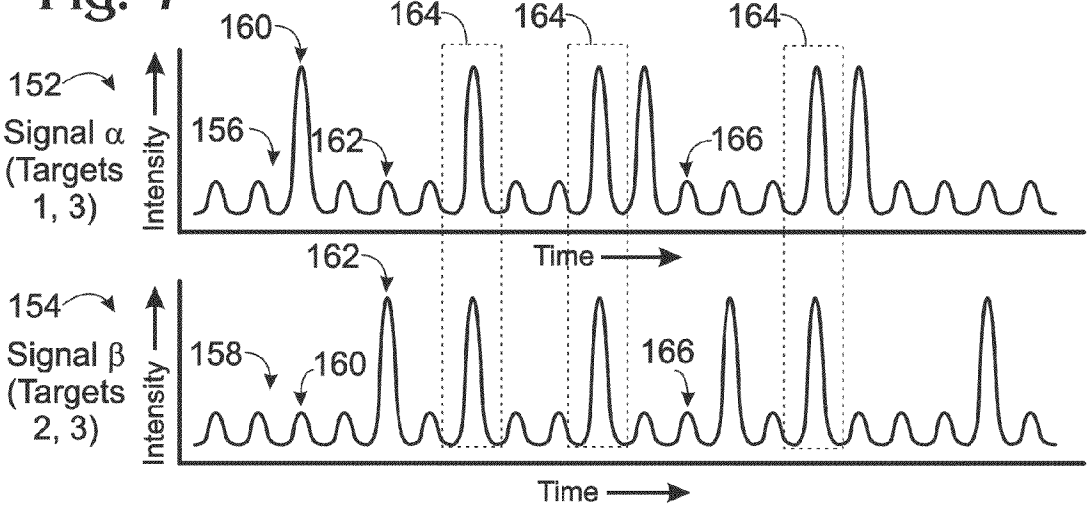



Fig. 8

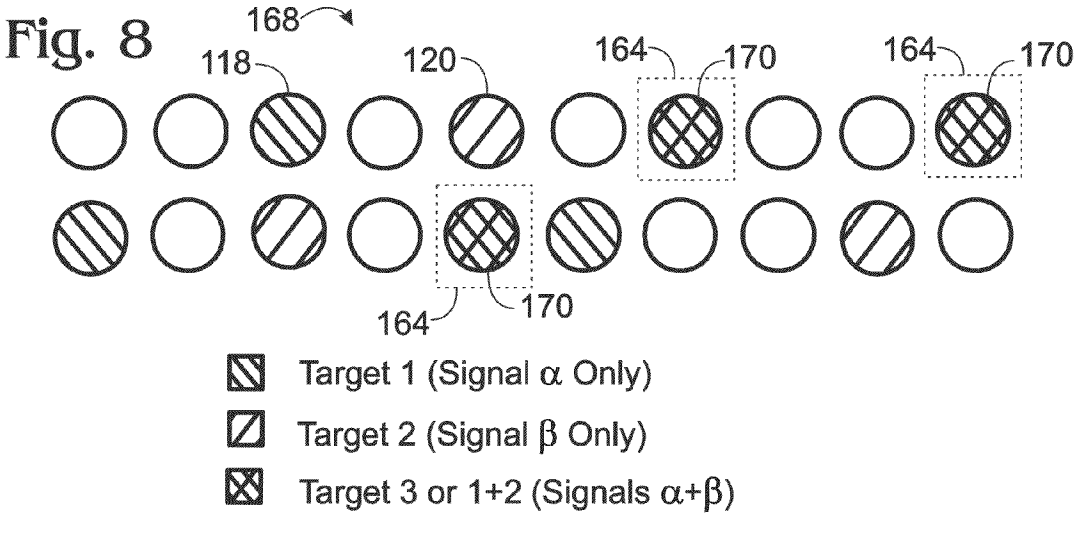


Fig. 9A

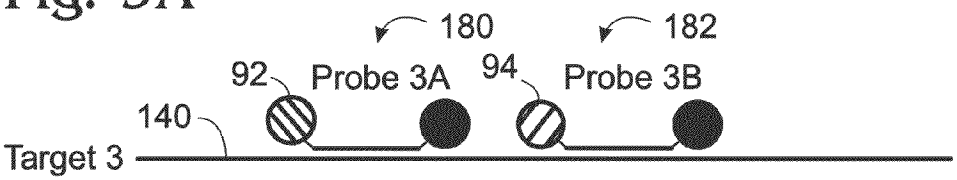



Fig. 9B

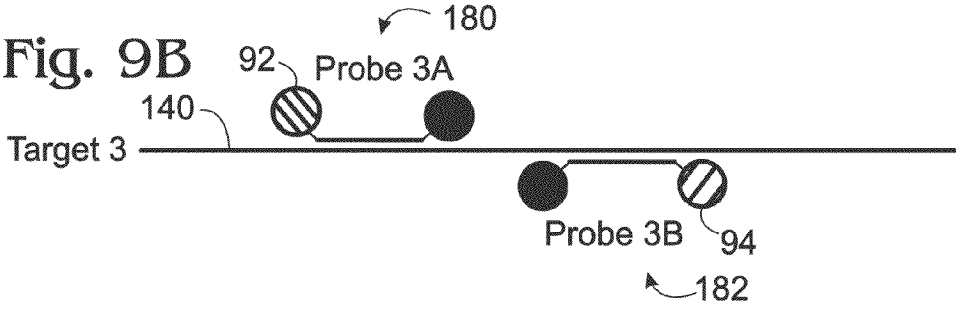



Fig. 10

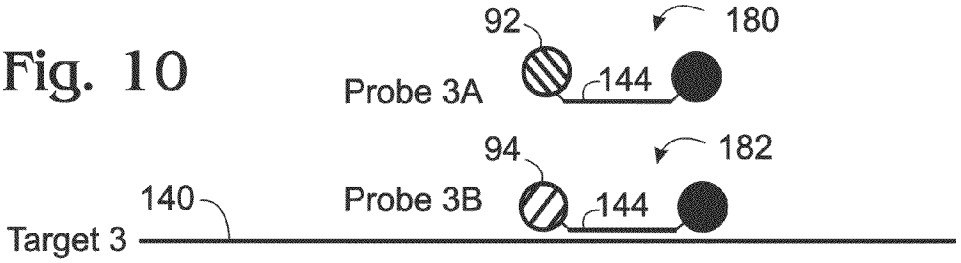



Fig. 11

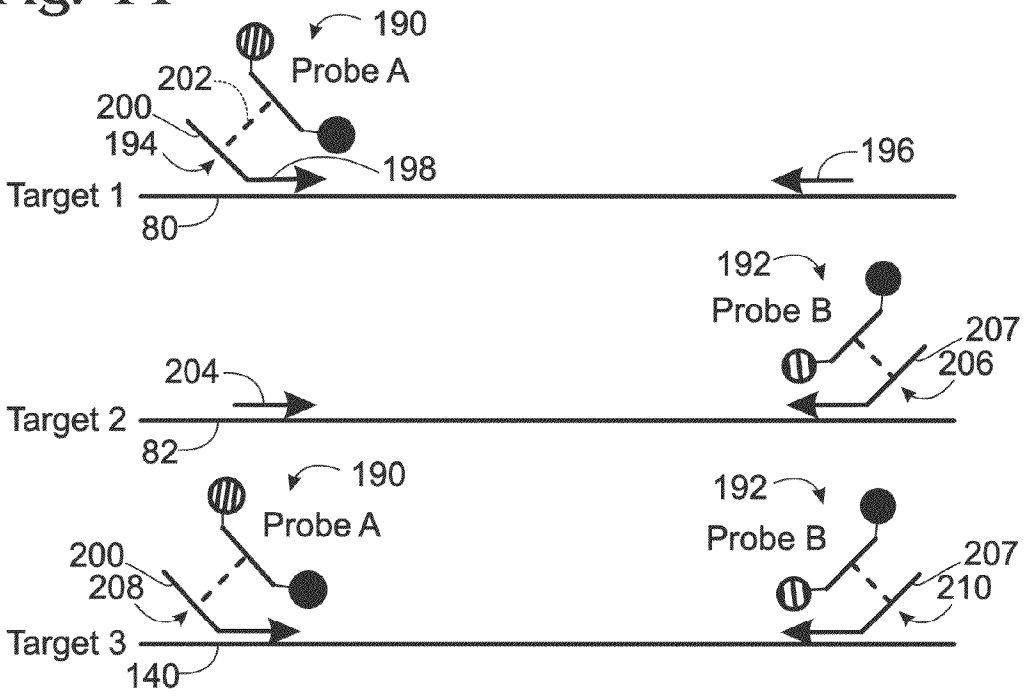


Fig. 12

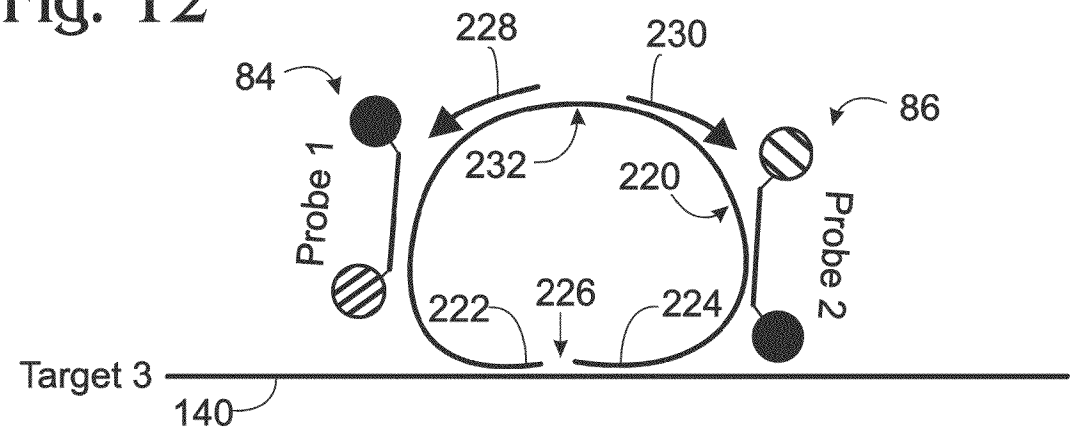

228
230
84
86
Probe 1
232
220
Probe 2
222
226
224
Target 3
140

Fig. 13

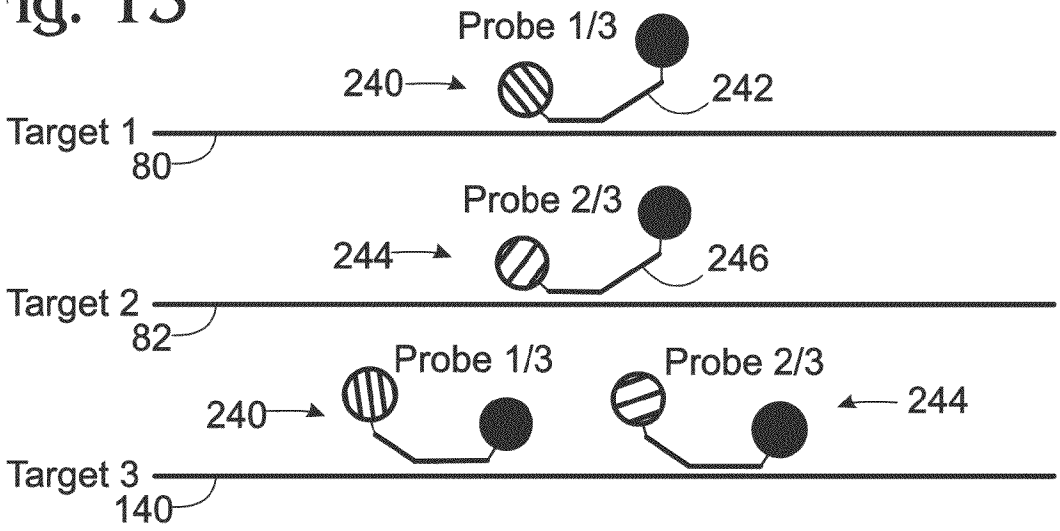

Probe 1/3
240
242
Target 1
80
Probe 2/3
244
246
Target 2
82
Probe 1/3
Probe 2/3
240
244
Target 3
140

Fig. 14

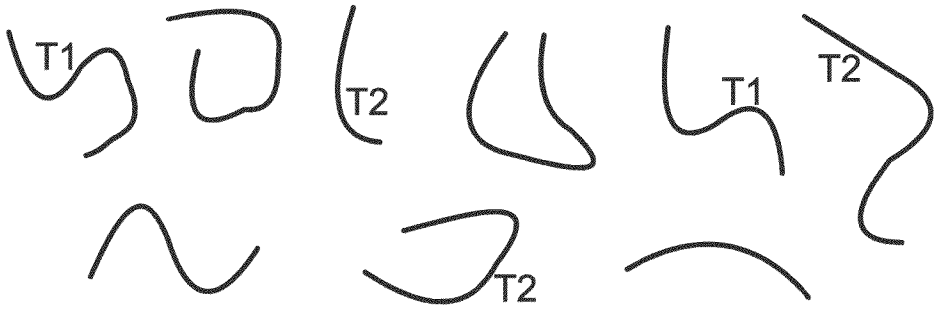



Fig. 15


Fig. 16

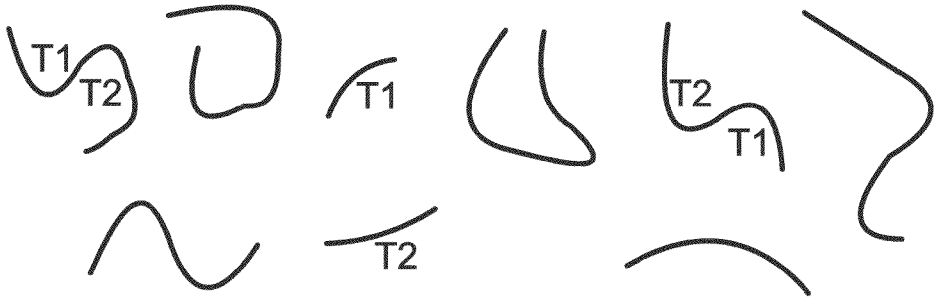


Fig. 17

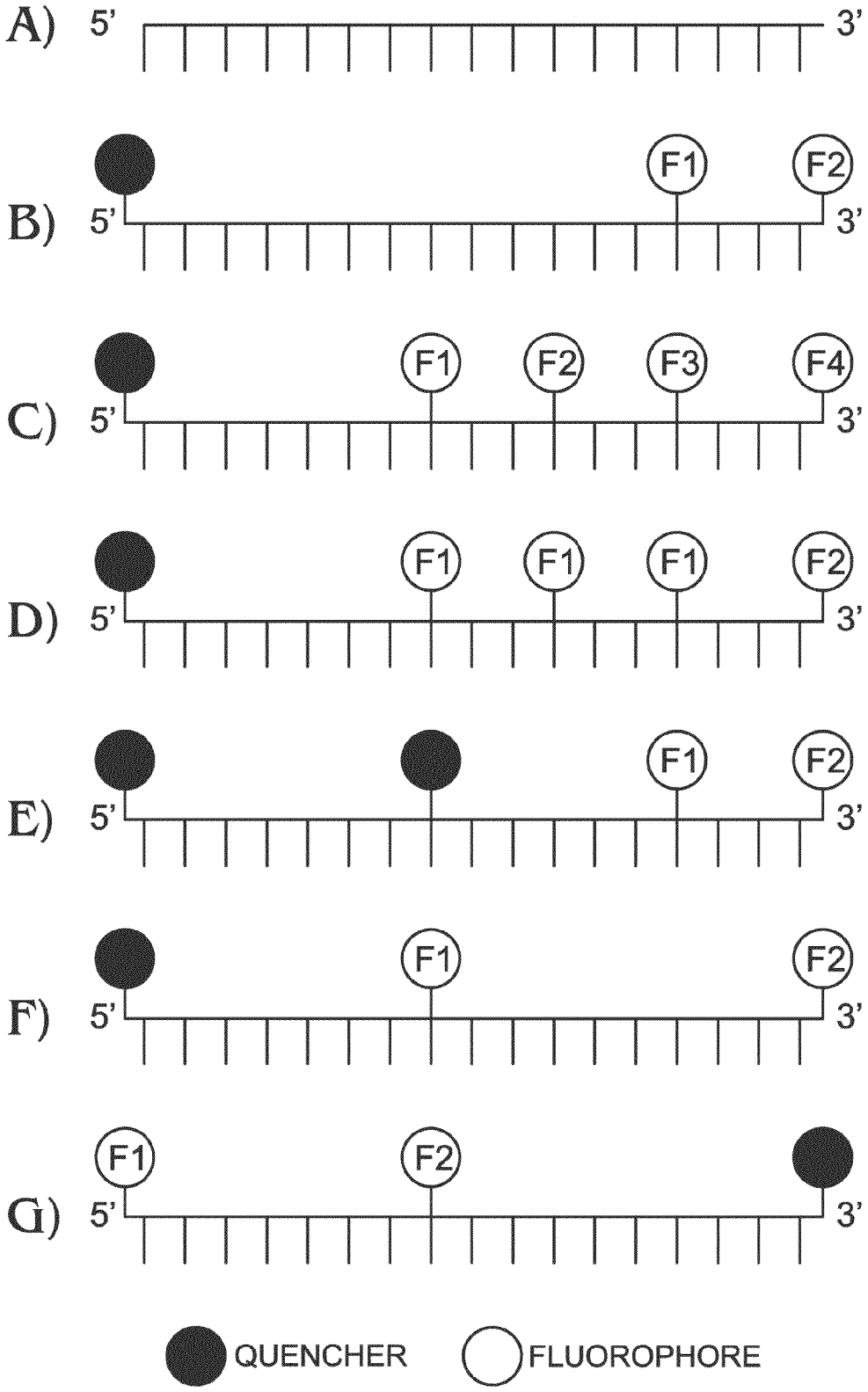

A)
5'
3'
B)
F1
F2
5'
3'
C)
F1
F2
F3
F4
5'
3'
D)
F1
F1
F1
F2
5'
3'
E)
F1
F2
5'
3'
F)
F1
F2
5'
3'
G)
F1
F2
5'
3'
QUENCHER
FLUOROPHORE

Fig. 18

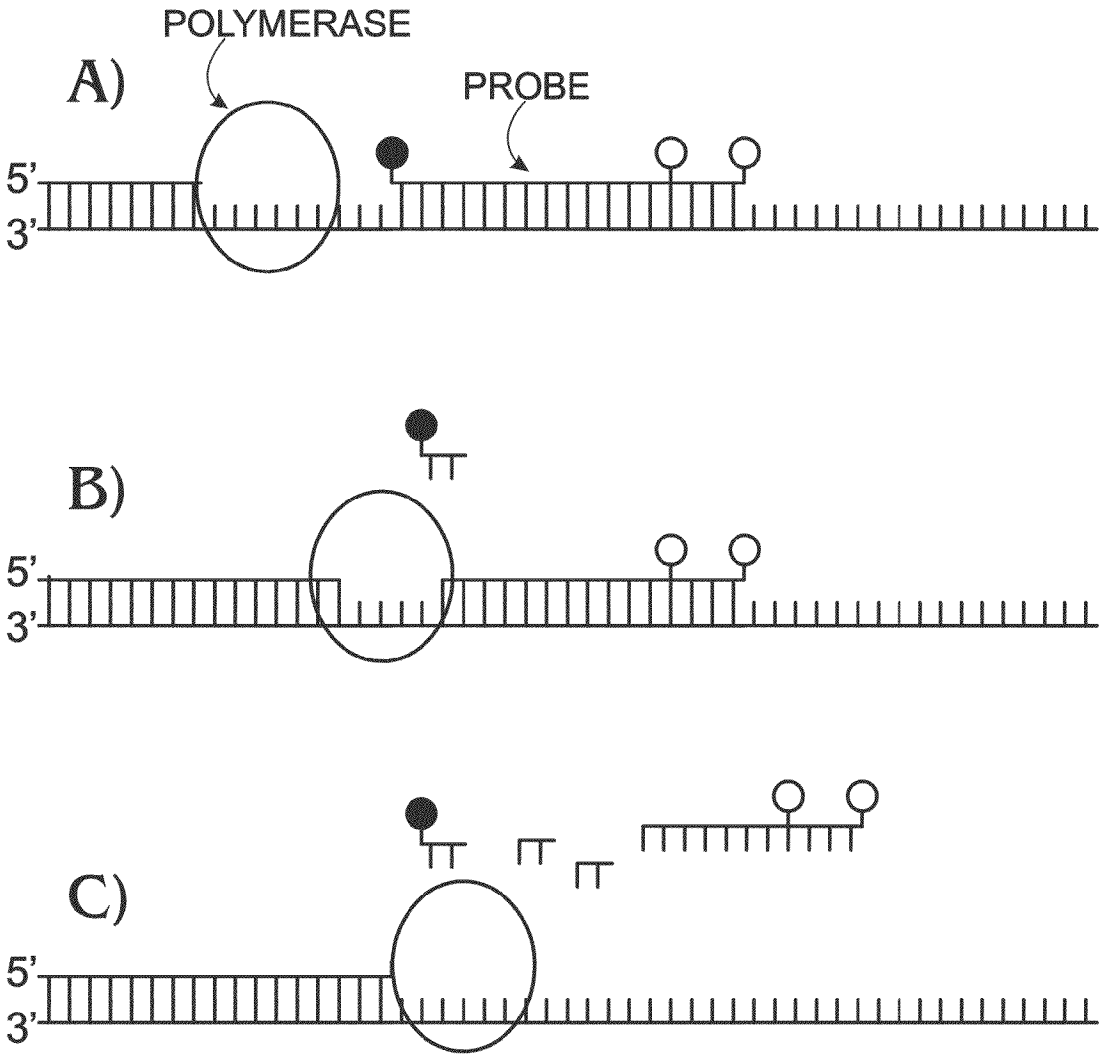


Fig. 19

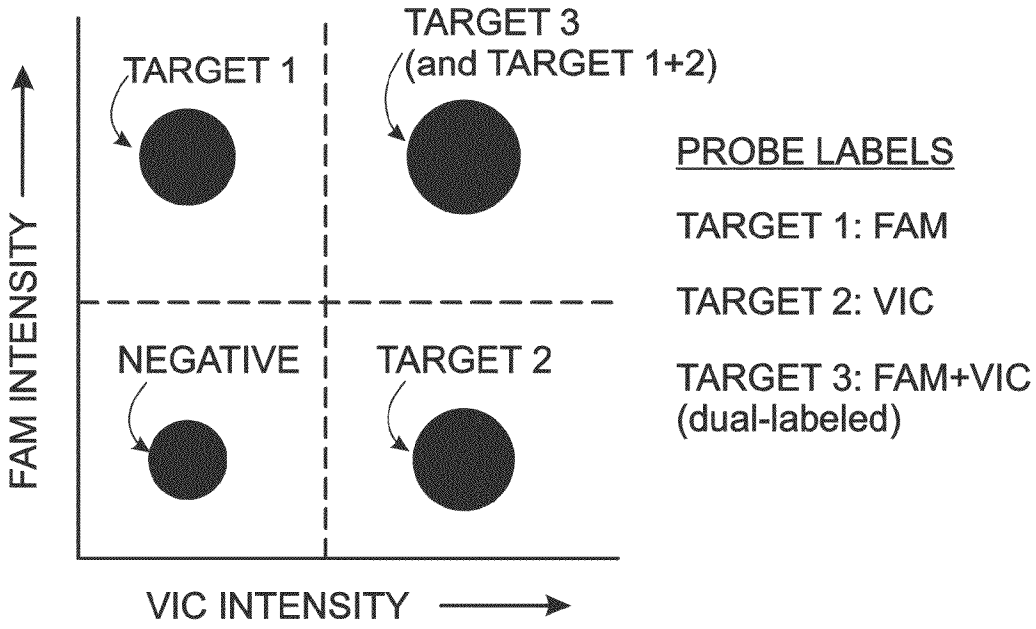

TARGET 1
TARGET 3
(and TARGET 1+2)
NEGATIVE
TARGET 2
FAM INTENSITY
VIC INTENSITY
PROBE LABELS
TARGET 1: FAM
TARGET 2: VIC
TARGET 3: FAM+VIC
(dual-labeled)

Fig. 20

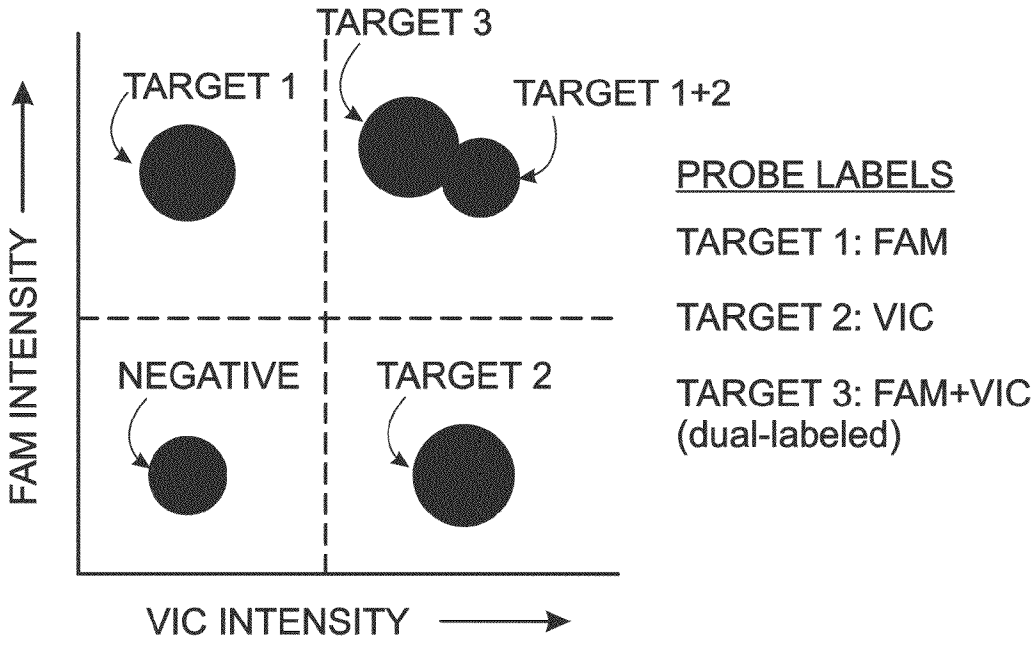

TARGET 1
TARGET 3
TARGET 1+2
NEGATIVE
TARGET 2
FAM INTENSITY
VIC INTENSITY
PROBE LABELS
TARGET 1: FAM
TARGET 2: VIC
TARGET 3: FAM+VIC
(dual-labeled)

Fig. 21

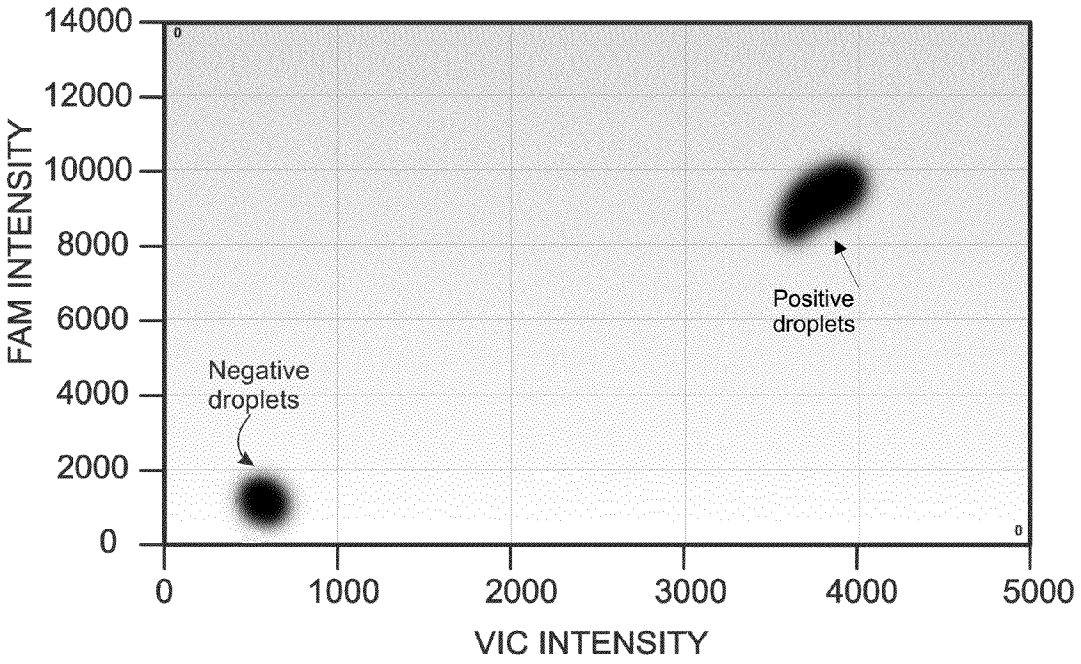
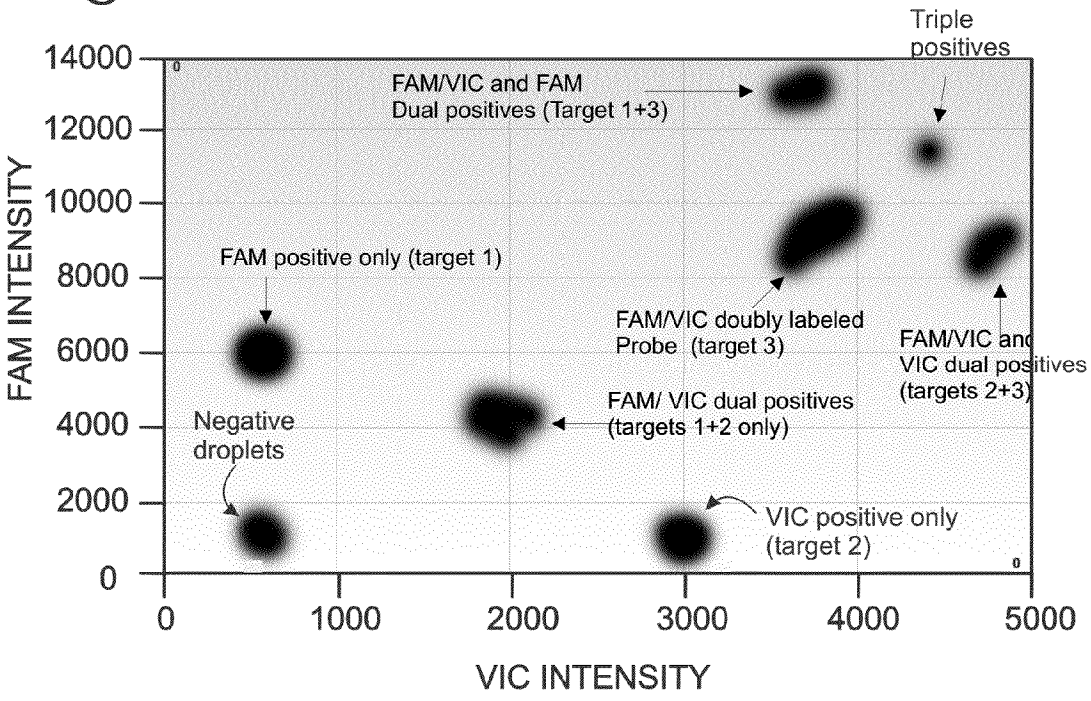

14000
12000
10000
8000
6000
4000
2000
0
FAM INTENSITY
0
1000
2000
3000
4000
5000
VIC INTENSITY
Positive droplets
Negative droplets

Fig. 22


14000
12000
10000
8000
6000
4000
2000
0
FAM INTENSITY
0
1000
2000
3000
4000
5000
VIC INTENSITY
Triple positives
FAM/VIC and FAM Dual positives (Target 1+3)
FAM positive only (target 1)
FAM/VIC doubly labeled Probe (target 3)
FAM/VIC and VIC dual positives (targets 2+3)
FAM/ VIC dual positives (targets 1+2 only)
Negative droplets
VIC positive only (target 2)

# MULTIPLEXED DIGITAL ASSAYS WITH COMBINATORIAL USE OF SIGNALS

## CROSS-REFERENCE TO PRIORITY APPLICATION

This application is based upon and claims the benefit under 35 U.S.C. §119(e) of U.S. Provisional Patent Application Ser. No. 61/454,373, filed Mar. 18, 2011, which is incorporated herein by reference in its entirety for all purposes.

## CROSS-REFERENCES

This application incorporates by reference in their entireties for all purposes the following materials: U.S. Pat. No. 7,041,481, issued May 9, 2006; U.S. Patent Application Publication No. 2010/0173394 A1, published Jul. 8, 2010; PCT Patent Application Publication No. WO 2011/120006 A1, published Sep. 29, 2011; PCT Patent Application Publication No. WO 2011/120024 A1, published Sep. 29, 2011; U.S. patent application Ser. No. 13/287,120, filed Nov., 1, 2011; U.S. Provisional Patent Application Ser. No. 61/507,082, filed Jul. 12, 2011; U.S. Provisional Patent Application Ser. No. 61/510,013, filed Jul. 20, 2011; and Joseph R. Lakowicz, PRINCIPLES OF FLUORESCENCE SPECTROSCOPY (2nd Ed. 1999).

## INTRODUCTION

Digital assays generally rely on the ability to detect the presence or activity of individual copies of an analyte in a sample. In an exemplary digital assay, a sample is separated into a set of partitions, generally of equal volume, with each containing, on average, less than about one copy of the analyte. If the copies of the analyte are distributed randomly among the partitions, some partitions should contain no copies, others only one copy, and, if the number of partitions is large enough, still others should contain two copies, three copies, and even higher numbers of copies. The probability of finding exactly 0, 1, 2, 3, or more copies in a partition, based on a given average concentration of analyte in the partitions, is described by a Poisson distribution. Conversely, the average concentration of analyte in the partitions may be estimated from the probability of finding a given number of copies in a partition.

Estimates of the probability of finding no copies and of finding one or more copies may be measured in the digital assay. Each partition can be tested to determine whether the partition is a positive partition that contains at least one copy of the analyte, or is a negative partition that contains no copies of the analyte. The probability of finding no copies in a partition can be approximated by the fraction of partitions tested that are negative (the "negative fraction"), and the probability of finding at least one copy by the fraction of partitions tested that are positive (the "positive fraction"). The positive fraction or the negative fraction then may be utilized in a Poisson equation to determine the concentration of the analyte in the partitions.

Digital assays frequently rely on amplification of a nucleic acid target in partitions to enable detection of a single copy of an analyte. Amplification may be conducted via the polymerase chain reaction (PCR), to achieve a digital PCR assay. The target amplified may be the analyte itself or a surrogate for the analyte generated before or after formation of the partitions. Amplification of the target can be detected optically with a fluorescent probe included in the reaction. In particular, the probe can include a dye that provides a fluorescence signal indicating whether or not the target has been amplified.



A digital PCR assay can be multiplexed to permit detection of two or more different targets within each partition. Amplification of the targets can be distinguished by utilizing target-specific probes labeled with different dyes, which produce fluorescence detected in different detection channels, namely, at different wavelengths or wavelength regions ("colors") of emission (and/or excitation). If a detector for a digital PCR assay can distinguishably measure the fluorescence emitted by R different dyes, then the assay is effectively capable of measuring R different targets. However, instruments with more detection channels, to detect more colors, are more expensive than those with fewer detection channels. Also, increasing the number of distinguishable dyes is expensive and becomes impractical beyond a certain number. On the other hand, many applications, especially where sample is limited, could benefit greatly from higher degrees of multiplexing.

A new approach is needed to increase the multiplex levels of digital assays.

## SUMMARY

The present disclosure provides a system, including methods, apparatus, and compositions, for performing a multiplexed digital assay on a greater number of targets through combinatorial use of signals.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a flowchart of an exemplary method of performing a digital assay, in accordance with aspects of the present disclosure.

FIG. **2** is a schematic view of an exemplary system for performing the digital assay of FIG. **1**, in accordance with aspects of the present disclosure.

FIG. **3** is a schematic view of a pair of targets and corresponding probes capable of reporting the presence or absence of target amplification via emitted light that may be detected to create a dedicated signal for each target in a digital amplification assay, in accordance with aspects of the present disclosure.

FIG. **4** is a pair of exemplary graphs of respective dedicated signals that may be created by detecting light emitted from the probes of FIG. **3** in a digital amplification assay performed in droplets, with each signal created from light detected over the same time interval from a fluid stream containing the droplets, in accordance with aspects of the present disclosure.

FIG. **5** is a schematic representation of how copies of the pair of targets of FIG. **3** are distributed among the droplets from which light is detected in FIG. **4**, based on the intensity of the respective dedicated signals of FIG. **4**, in accordance with aspects of the present disclosure.

FIG. **6** is a schematic view of three targets and corresponding exemplary probes capable of reporting the presence or absence of target amplification via emitted light that may be detected to create a pair of composite signals in a digital amplification assay, in accordance with aspects of the present disclosure.

FIG. **7** is a pair of exemplary graphs of a pair of composite signals that may be created by detecting fluorescence emission from the three probes of FIG. **6** in a digital amplification assay performed in droplets, with emitted light detected in two different wavelength regimes over the same time interval

from a fluid stream containing the droplets, in accordance with aspects of the present disclosure.

FIG. **8** is a schematic representation of how copies of the three targets of FIG. **6** are distributed among the droplets from which light is detected in FIG. **7**, based on the intensity of the respective composite signals of FIG. **7**, in accordance with aspects of the present disclosure.

FIG. **9**A is a schematic view of the third target of FIG. **6** and another exemplary probe configuration capable of reporting the presence or absence of third target amplification via emitted light, which may be used in conjunction with the first and second target probes of FIG. **6** to create only a pair of composite signals representing amplification of the three targets in a digital amplification assay, in accordance with aspects of the present disclosure.

FIG. **9**B is a schematic view of the third target of FIG. **6** and yet another exemplary probe configuration specific for the third target, which may be used in conjunction with the first and second target probes of FIG. **6** to create only a pair of composite signals representing the three targets in a digital amplification assay, in accordance with aspects of the present disclosure.

FIG. **10** is a schematic view of the third target of FIG. **6** and still another exemplary probe configuration capable of reporting the presence or absence of third target amplification via emitted light, which may be used in conjunction with the first and second target probes of FIG. **6** to create only a pair of composite signals representing amplification of the three targets in a digital amplification assay, in accordance with aspects of the present disclosure.

FIG. **11** is a schematic view of three targets and corresponding exemplary primers that enable use of only two probes to report amplification of the three targets in a digital amplification assay, in accordance with aspects of the present disclosure.

FIG. **12** is a schematic view of the third target of FIG. **6** and another exemplary probe and primer configuration that enables use of only two probes to report amplification of three targets in a digital amplification assay, in accordance with aspects of the present disclosure.

FIG. **13** is a schematic view of the three targets of FIG. **6** with yet another exemplary configuration of only two probes that enables use of the two probes to report amplification of three targets in a digital amplification assay, in accordance with aspects of the present disclosure.

FIG. **14** is a schematic view of a population of fragments containing a pair of unlinked targets, T**1** and T**2**, in accordance with aspects of the present disclosure.

FIG. **15** is a schematic view of a population of fragments taken as in FIG. **14**, but with the pair of targets always linked to each other on the same individual fragments, in accordance with aspects of the present disclosure.

FIG. **16** is a schematic view of a population of fragments taken as in FIG. **14**, but with the pair of targets only partially linked to each other within the population, in accordance with aspects of the present disclosure.

FIG. **17** is a schematic representation of a set of exemplary multi-labeled probes for use in digital amplification assays, in accordance with aspects of the present disclosure.

FIG. **18** is a schematic illustration of a template molecule being copied by DNA polymerase during target amplification in the presence of a multi-labeled probe molecule and depicting probe degradation by the polymerase to separate a quencher from fluorophores of the probe molecule, in accordance with aspects of the present disclosure.

FIG. **19** is an exemplary two-dimensional histogram of droplet intensities, showing clusters that may be obtained in a multiplexed digital amplification assay for three targets performed with a combination of single-labeled and dual-labeled probes each labeled with FAM, VIC, or both FAM and VIC, in accordance with aspects of the present disclosure.

FIG. **20** is another exemplary two-dimensional histogram of droplet intensities, showing clusters that may be obtained in the assay of FIG. **19**, with partial resolution of the cluster for target-1+2-positive droplets from the cluster for target-3-positive droplets, in accordance with aspects of the present disclosure.

FIG. **21** is an exemplary two-dimensional intensity histogram of droplet intensities, showing clusters that may be obtained in a digital amplification assay performed with only a multi-labeled FAM, VIC probe, in accordance with aspects of the present disclosure.

FIG. **22** is another exemplary two-dimensional intensity histogram of droplet intensities, showing clusters that may be obtained in a digital amplification assay performed as in FIG. **21**, but with the assay supplemented with pair of single-labeled FAM or VIC probes in addition to the multi-labeled FAM, VIC probe, in accordance with aspects of the present disclosure.

## DETAILED DESCRIPTION

The present disclosure provides a system, including methods, apparatus, and compositions, for performing a multiplexed digital assay on a greater number of targets through combinatorial use of signals. The method may be described as a color-based approach to multiplexing.

A method of performing a multiplexed digital amplification assay, such as a PCR assay, is provided. In the method, more than R targets may be amplified in partitions. R signals may be created. The signals may be representative of light detected in R different wavelength regimes from the partitions, where R≥2. An average level of each target in the partitions may be calculated based on the R signals, with the level calculated accounting for a coincidence, if any, of different targets in the same individual partitions.

Another method of performing a multiplexed digital amplification assay is provided. In the method, more than R targets may be amplified in droplets. R signals may be created, with the signals representative of light detected in R different wavelength regimes from the droplets, where R≥2. An average level of each of the more than R targets may be calculated by finding solutions to a set of simultaneous equations.

Yet another method of performing a multiplexed digital amplification assay is provided. In the method, R targets may be amplified in droplets. R signals may be created, where R≥2, with the signals representative of light detected in R different wavelength regimes from the droplets. Each of the signals may report amplification of a different combination of at least two of the targets. An average level of each target in the droplets may be calculated based on the R signals, without determining which of the at least two targets for each signal amplified in individual amplification-positive droplets for such signal.

A composition is provided. The composition may comprise a droplet containing a probe. The probe may include an oligonucleotide, a first fluorophore, a second fluorophore, and an energy transfer moiety. The energy transfer moiety may be a quencher and/or an energy transfer partner for one or both of the first and second fluorophores.

Further aspects of the present disclosure are presented in the following sections: (I) system overview, and (II) examples.

## I. SYSTEM OVERVIEW

FIG. **1** shows a flowchart of an exemplary method **40** of performing a digital assay. The steps presented for method **40** may be performed in any suitable order and in any suitable combination. Furthermore, the steps may be combined with and/or modified by any other suitable steps, aspects, and/features of the present disclosure.

A sample may be prepared for the assay, indicated at **42**. Preparation of the sample may include any suitable manipulation of the sample, such as collection, dilution, concentration, purification, lyophilization, freezing, extraction, combination with one or more assay reagents, performance of at least one preliminary reaction to prepare the sample for one or more reactions in the assay, or any combination thereof, among others. Preparation of the sample may include rendering the sample competent for subsequent performance of one or more reactions, such as one or more enzyme catalyzed reactions and/or binding reactions.

In some embodiments, preparation of the sample may include combining the sample with reagents for amplification and for reporting whether or not amplification occurred. Such reagents may include any combination of primers for the targets (e.g., a forward primer and a reverse primer for each target), reporters (e.g., probes) for detecting amplification of the targets, dNTPs and/or NTPs, at least one enzyme (e.g., a polymerase, a ligase, a reverse transcriptase, or a combination thereof, each of which may or may not be heat-stable), or the like. Accordingly, preparation of the sample may render the sample (or partitions thereof) capable of amplification of each of one or more targets, if present, in the sample (or a partition thereof).

The sample may be separated into partitions, indicated at **44**. Separation of the sample may involve distributing any suitable portion or all of the sample to the partitions. Each partition may be and/or include a fluid volume that is isolated from the fluid volumes of other partitions. The partitions may be isolated from one another by a carrier fluid, such as a continuous phase of an emulsion, by a solid phase, such as at least one wall of a container, or a combination thereof, among others. In some embodiments, the partitions may be droplets disposed in a continuous phase, such that the droplets and the continuous phase collectively form an emulsion.

The partitions may be formed by any suitable procedure, in any suitable manner, and with any suitable properties. For example, the partitions may be formed with a fluid dispenser, such as a pipette, with a droplet generator, by agitation of the sample (e.g., shaking, stirring, sonication, etc.), or the like. Accordingly, the partitions may be formed serially, in parallel, or in batch. The partitions may have any suitable volume or volumes. The partitions may be of substantially uniform volume or may have different volumes. Exemplary partitions having substantially the same volume are monodisperse droplets. Exemplary volumes for the partitions include an average volume of less than about 100, or 1 μL, less than about 100, 10, or 1 nL, or less than about 100, 10, or 1 pL, among others.

The partitions, when formed, may be competent for performance of one or more reactions in the partitions. Alternatively, one or more reagents may be added to the partitions after they are formed to render them competent for reaction. The reagents may be added by any suitable mechanism, such as a fluid dispenser, fusion of droplets, or the like. Any of the reagents may be combined with the partitions (or a bulk phase sample) in a macrofluidic or microfluidic environment.

One or more reactions may be performed in the partitions, indicated at **46**. Each reaction performed may occur selectively (and/or substantially) in only a subset of the partitions, such as less than about one-half, one-fourth, or one-tenth of the partitions, among others. The reaction may involve a target, which may, for example, be a template and/or a reactant (e.g., a substrate), and/or a binding partner, in the reaction. The reaction may occur selectively (or selectively may not occur) in partitions containing at least one copy of the target.

The reaction may or may not be an enzyme-catalyzed reaction. In some examples, the reaction may be an amplification reaction, such as a polymerase chain reaction and/or ligase chain reaction. Accordingly, a plurality of amplification reactions for a plurality of targets may be performed simultaneously in the partitions.

Performing a reaction may include subjecting the partitions to one or more conditions that promote occurrence of the reaction. The conditions may include heating the partitions and/or incubating the partitions at a temperature above room temperature. In some examples, the conditions may include thermally cycling the partitions to promote a polymerase chain reaction and/or ligase chain reaction.

R signals may be created that are representative of light detected from the partitions, indicated at **48**. The R signals may be 2, 3, 4, or more signals. In some examples, light corresponding to each signal may be detected with a distinct sensor, and/or light corresponding to at least two signals may be detected at different times with the same sensor. The R signals may correspond to light detected in respective wavelength regimes that are different from one another. Each wavelength regime may be characterized by a wavelength(s) or and/or wavelength range(s) at which the partitions are illuminated (e.g., with excitation light) and/or a wavelength(s) or and/or wavelength range(s) at which light from the partitions is detected (e.g., emitted light). The light detected may be light emitted from one or more fluorophores.

Each of the R signals may be created in a distinct detection channel. Accordingly, R signals may be created in R detection channels.

Each signal may be a composite signal that represents two, three, four, or more reactions/assays and thus two, three, four, or more targets of the reactions/assays. The composite signal may include two or more integral signal portions that each represent a different reaction/assay and target. Analysis of one of the composite signals by itself, without the benefit of data from the other composite signals, may (or may not) permit estimation of a collective concentration, but not individual concentrations, for two or more targets represented by the composite signal. Instead, as described further below, analysis of the composite signals together permits calculation of the concentration of each target. (The terms "estimation" and "calculation" are used interchangeably.)

The R composite signals (and/or R detection channels) may represent more than R reactions and/or targets, with the number of reactions/assays and targets depending on configuration. For example, the R signals may be or include two composite signals (arbitrarily termed α and β) collectively representing three reactions/assays and/or three targets (arbitrarily termed **1**, **2**, and **3**), with each composite signal representing a different combination of two reactions/assays/targets (e.g., targets **1** and **2** for α and targets **1** and **3** for β). Alternatively, the R signals may be three composite signals (arbitrarily termed α, β, and γ) collectively representing up to seven reactions/assays/targets (**1** to **7**), if each composite sig-

nal represents a different combination of up to four reactions/assays/targets each (e.g., targets **1**, **2**, **3**, and **4** for α; targets **2**, **4**, **5**, and **6** for β; and targets **3**, **4**, **6**, and **7** for γ). The R signals may be four composite signals representing up to fifteen reactions/assays/targets, if each composite signal represents a different combination of up to eight reactions/assays/targets each.

More generally, $2^R-1$ targets can be assayed with R composite signals (or wavelength regimes). To assay $2^R-1$ targets in R wavelength regimes, each target may be represented by a different wavelength regime or combination of wavelength regimes than every other target. A set of $2^R-1$ targets can be represented and assayed when all of the wavelength regimes have been utilized individually and in all possible combinations.

Each composite signal may be created based on detected light emitted from one or more probes in the partitions. The one or more probes may report whether at least one of two or more particular reactions represented by the signal has occurred in a partition and thus whether at least one copy of at least one of two or more particular targets corresponding to the two or more particular reactions is present in the partition. The intensity of a composite signal corresponding to the probes may be analyzed to determine whether or not at least one of the particular reactions has occurred and at least one copy of one of the particular targets is present. The intensity may vary among the partitions according to whether at least one of the particular reactions occurred or did not occur (e.g., above a threshold extent) and at least one of the particular targets is present in or absent from each individual partition.

The probes represented by a composite signal may include different fluorophores. In other words, light emitted from different fluorophores may be detected to create at two different integral portions of the composite signal for a particular wavelength regime. Alternatively, or in addition, the same fluorophore may be included in one probe or two or more probes for at least two targets represented by the composite signal. In some cases, the same fluorophore may be included in a probe for each target represented by the composite signal.

Each probe may include a nucleic acid (e.g., an oligonucleotide) and at least one fluorophore. Different probes with different oligonucleotide sequences and/or different fluorophores (or fluorophore combinations) may be used to create at least two different integral portions of the signal. Alternatively, or in addition, the same probe may be used as a reporter for at least two different targets represented by the composite signal (e.g., see Examples 3-5). In some cases, the same probe may be used as a reporter for each target represented by the composite signal.

The composite signal detected from each partition, and the partition itself, may be classified as being positive or negative for the reactions/assays/targets represented by the signal or corresponding wavelength regime. Classification may be based on the strength of the signal. If the signal/partition is classified as positive, at least one of the reactions/assays represented by the signal is deemed to have occurred and at least one copy of at least one of the targets represented by the signal is deemed to be present in the partition. In contrast, if the signal/partition is classified as negative, none of the reactions/assays represented by the signal is deemed to have occurred and no copy of any of the targets represented by the signal is deemed to be present in the partition.

The composite signals collectively permit estimation of target concentrations by representation of a different combination of targets in each detection channel. Accordingly, each target, when present without any of the other targets in a partition, may produce a unique target signature among the wavelength regimes. For example, some of the targets, if present alone in a partition, may selectively change the signal strength for only one wavelength regime. Others of the targets, if present alone in a partition, may selectively change the signal strength for a unique combination of two of the wavelength regimes, still other targets may selectively change the signal strength for a unique combination of three of the wavelength regimes, and so on, optionally up to the number of wavelength regimes/detection channels.

A fraction of the partitions may have a coincidence of different targets, where each of these partitions contains a copy of each of two or more targets in the same individual partitions. Moreover, each of these partitions may contain a copy of each of two or more distinct targets, which, for a particular partition, collectively may produce a signature that is the same as that of a target not present in the partition. However, the fraction of partitions containing two or more distinct targets may (or may not) be kept relatively low, by working in a dilute regime, such as with less than about one-half, one-fifth, or one-tenth, among others, of the partitions containing more than one target molecule when the partitions are formed. In any event, a suitable estimation of concentration, as described below, may take into account the occurrence of two or more target molecules, representing the same target or different targets, in the same individual partitions. Alternatively, if working in a sufficiently dilute regime, the occurrence of two or more target molecules per partition may be sufficiently rare to ignore for a desired accuracy of concentration.

A number of partitions that are positive may be determined for each signal, indicated at **50**. For example, a number of partitions that are positive only for each particular composite signal or corresponding wavelength regime/detection channel may be determined individually (e.g., counted) for each signal or channel (i.e., a number for each channel). Also, a number of partitions that are positive only for each particular combination (or at least one combination or each of two or more combinations) of composite signals or corresponding wavelength regimes may be determined individually (e.g., counted) for each combination of signals or channels (i.e., a number for each combination, and particularly each combination corresponding to a particular target).

A distinct fraction of the partitions positive for each signal alone and for each signal combination may be determined. The fraction for each signal or signal combination may be determined by dividing the number of partitions for the signal/combination, determined at **50**, by the total number of partitions from which signals are detected. The total number of partitions may be counted or estimated.

A level of each target may be calculated, indicated at **52**. The level may be an average level, such as an average concentration of molecules of the target per partition. Generally, if R signals are detected from the partitions in R wavelength regimes, the average level of each of more than R targets (e.g., up to $2^R-1$ targets) may be calculated. The level of each target may be calculated based on the respective numbers of partitions positive for each signal alone and signal combination. The calculation may be based on copies of each target having a Poisson distribution among the partitions. The concentrations may, for example, be calculated by finding solutions to a series of simultaneous equations (interchangeably termed a set of simultaneous equations), each having the same variables. The simultaneous equations may be linear equations. Alternatively, or in addition, each equation may contain at least $2^R-1$ variables. The solutions may be found by numerical analysis, also termed numerical approximation. Further

aspects of calculating average target levels are described elsewhere in the present disclosure, such as in Examples 6 and 7.

FIG. **2** shows an exemplary system **60** for performing the digital assay of FIG. **1**. System **60** may include a partitioning assembly, such as a droplet generator **62** ("DG"), a thermal incubation assembly, such as a thermocycler **64** ("TC"), a detection assembly (a detector) **66** ("DET"), and a data processing assembly (a processor) **68** ("PROC"), or any combination thereof, among others. The data processing assembly may be, or may be included in, a controller that communicates with and controls operation of any suitable combination of the assemblies. The arrows between the assemblies indicate optional movement or transfer, such as movement or transfer of fluid (e.g., a continuous phase of an emulsion) and/or partitions (e.g., droplets) or signals/data. Any suitable combination of the assemblies may be operatively connected to one another, and/or one or more of the assemblies may be unconnected to the other assemblies, such that, for example, material/data is transferred manually.

System **60** may operate as follows. Droplet generator **62** may form droplets disposed in a carrier fluid, such as a continuous phase. The droplets may be cycled thermally with thermocycler **64** to promote amplification of targets in the droplets. Composite signals may be detected from the droplets with detector **66**. The signals may be processed by processor **68** to calculate levels of the targets.

Further aspects of sample preparation, droplet generation, assays, reagents, reactions, thermal cycling, detection, and data processing, among others, that may be suitable for the methods and systems disclosed herein, are described below and in the documents listed above under Cross-References, which are incorporated herein by reference, particularly U.S. Patent Application Publication No. 2010/0173394 A1, published Jul. 8, 2010; PCT Patent Application Publication No. WO 2011/120006 A1, published Sep. 29, 2011; PCT Patent Application Publication No. WO 2011/120024 A1, published Sep. 29, 2011; and U.S. patent application Ser. No. 13/287,120, filed Nov., 1, 2011.

## II. EXAMPLES

This section presents selected aspects and embodiments of the present disclosure related to methods and compositions for performing multiplexed digital assays. These aspects and embodiments are included for illustration and are not intended to limit or define the entire scope of the present disclosure.

### Example 1

### Digital Amplification Assays with Dedicated Signals and Composite Signals

This example compares and contrasts exemplary digital amplification assays utilizing (i) a pair of dedicated signals for two targets, see FIGS. **3-5**, and (ii) a pair of composite signals for three targets, see FIGS. **6-8**. The principles explained here may be extended to R signals for $2^R-1$ targets.

FIG. **3** shows a pair of nucleic acid targets **80**, **82** ("Target **1**" and "Target **2**") and corresponding probes **84**, **86** ("Probe **1**" and "Probe **2**") that may be used to create a dedicated signal for amplification of each target in a digital amplification assay. Each probe may include an oligonucleotide **88**, **90**, a fluorophore **92**, **94**, and a quencher **96**. The fluorophore(s) and quencher are associated with and/or attached to the oligonucleotide, such as attached covalently. The probe also or alternatively may include a binding moiety (a minor groove binder) for the minor groove of a DNA duplex, which may be conjugated to the oligonucleotide and may function to permit a shorter oligonucleotide to be used in the probe. The probe may be a TaqMan probe, a molecular beacon probe, a scorpion probe, a locked nucleic acid probe, or the like.

Each oligonucleotide may provide target specificity by hybridization predominantly or at least substantially exclusively to an amplicon produced by amplification of only one of the two targets. Hybridization of the oligonucleotide to its corresponding target/amplicon is illustrated schematically at **98**.

Fluorophores **92**, **94** may be optically distinguishable from each other, as illustrated schematically by a distinct hatch pattern for each fluorophore. For example, the fluorophores may have distinct absorption spectra and/or maxima, and/or distinct emission spectra and/or emission maxima. Proper selection of the wavelength regime used for detection allows the fluorophores to be distinguished. In other words, the wavelength or wavelength band of excitation light used for each wavelength regime and/or the wavelength or wavelength band of emitted light received and sensed by the sensor for the wavelength regime provides selective detection of light from only one of the fluorophores in the detection channel. Exemplary fluorophores that may be suitable include FAM, VIC, HEX, ROX, TAMRA, JOE, etc.

Quencher **96** is configured to quench the signal produced by fluorophore **92** or **94** in a proximity-dependent fashion. Light detected from the fluorophore may increase when the associated oligonucleotide **88** or **90** binds to the amplified target, to increase the separation distance between the fluorophore and the quencher, or when the probe is cleaved during target amplification, among others. In some cases, the quencher may be replaced by, or supplemented with, a fluorophore that is capable of energy transfer with fluorophore **92** or **94**.

FIG. **4** shows a pair of exemplary graphs **102**, **104** of data collected in an exemplary digital amplification assay for Target **1** and Target **2** performed in droplets. Each graph plots a dedicated signal **106** ("Signal **1**") or signal **108** ("Signal **2**") that represents light detected from respective probes **84**, **86** (and/or one or more modified (e.g., cleavage) products thereof) (see FIG. **3**). Each dedicated signal is created from light detected over the same time period from a fluid stream containing the droplets and flowing through an examination region of a channel. Signal **1** reports whether or not Target **1** is present in each droplet, and Signal **2** reports whether or not Target **2** is present in each target. In particular, if the strength of Signal **1** (or Signal **2**) increases above a threshold **110**, then Target **1** (or Target **2**) is deemed to be present (and amplified) in a corresponding droplet. In the present illustration, each droplet, whether positive or negative for each target, produces an increase in signal strength above the baseline signal that forms an identifiable peak **112**. Accordingly, each signal may vary in strength with the presence or absence of a droplet and with the presence or absence of a corresponding target.

Each target is present here at an average level (or frequency) of about 0.2 in the droplets. In other words, each target is amplified and detected on average about once every five droplets. Accordingly, the expected frequency of droplets containing both targets is the product of the two droplet frequencies, or about 0.04 (1 out of every 25 droplets). Consistent with this frequency, a droplet that is positive for both targets is present only once on the twenty droplets analyzed here, and is indicated by a dashed box at **114** extending around the signal peaks for the droplet in graphs **102**, **104**.

FIG. **5** schematically represents the distribution of Targets **1** and **2** in a set of droplets **116** corresponding to and in the

same order as the droplet signal peaks of FIG. **4**. Droplets positive for Signal **1**, such as the droplet indicated at **118**, are hatched according to fluorophore **92**, and droplets positive for Signal **2**, such as the droplet indicated at **120**, are hatched according to fluorophore **94** (see FIG. **3**). A double-positive droplet **122** containing both Target **1** and Target **2** is double-hatched and indicated by dashed box **114**.

FIG. **6** shows three targets **80**, **82**, and **140** and corresponding exemplary probes **84**, **86**, and **142**, respectively, that may be used to create a pair of composite signals for the three targets in a digital amplification assay. Two of the targets and probes, namely, targets **80** and **82** (Target **1** and Target **2**) and probes **84** and **86** (Probe **1** and Probe **2**) are the same targets and probes shown and utilized in FIGS. **3-5**. Target **140** (Target **3**) and its corresponding probe **142** (Probe **3**) may be introduced into the multiplexed assay of FIGS. **3-5** to increase the level of multiplexing and the amount of target information that can be extracted from the assay without increasing the number of detection channels.

Amplification of Target **3** is reported by Probe **3**. The probe includes an oligonucleotide **144** that hybridizes specifically to Target **3** (and/or an amplicon thereof), relative to Targets **1** and **2**. The probe may be double-labeled with the same fluorophores (**92**, **94**) present individually in Probe **1** and Probe **2** for reporting respective Target **1** and Target **2** amplification. The probes for the three targets may be selected to permit detection of target amplification in only two detection channels, rather than the three detection channels that would be necessary with the use of a dedicated detection channel for each target. Examples 2-5 describe other exemplary probe configurations that may be suitable to increase the level of multiplexing.

FIG. **7** shows a pair of exemplary graphs **152**, **154** of a pair of composite signals **156**, **158** that may be detected in a pair of wavelength regimes/detection channels. The composite signals, arbitrarily designated α and β, are representative of light detected from the three probes of FIG. **6** in a digital amplification assay performed in droplets. Each composite signal is created from light detected over the same time period from a fluid stream containing the droplets. To simplify the presentation, Target **1** and Target **2** are present at the same frequency and in the same droplets as in FIGS. **4** and **5**.

Each composite signal, α or β (**156** or **158**), represents a pair of targets. Signal a (graph **152**) has a strength for each droplet that indicates whether the droplet is positive or negative for at least one member of a first pair of targets, namely, Target **1** and Target **3**. Signal β (graph **154**) has a strength for each droplet that indicates whether the droplet is positive or negative for at least one member of a different second pair of targets, namely, Target **2** and Target **3**. Accordingly, each composite signal analyzed by itself may provide no information about how frequently a given member of the pair of targets is present in droplets.

The composite signals analyzed in combination provide additional information about target frequency that cannot be deduced from the composite signals in isolation from one another. Each target, when present without other targets in a droplet, produces a signal signature that is distinct from the signatures of each other target individually. The signature for Target **1** in a droplet is indicated at **160**: positive for Signal α and negative for Signal β. The signature for Target **2** in a droplet is indicated at **162**: negative for Signal α and positive for Signal β. Furthermore, the signature for Target **3** in a droplet is outlined by dashed boxes at **164**: positive for both Signal α and Signal β. Finally, the signature for none of Targets **1**, **2**, and **3** in a droplet is indicated at **166**: negative for both Signal α and Signal β.

FIG. **8** schematically represents the distribution of Targets **1**, **2**, and **3** in a set of droplets **168** corresponding to and in the same order as the droplet signal peaks of FIG. **7**. Single-positive droplets that are positive for Signal α only, such as the droplet indicated at **118**, are hatched according to fluorophore **92**, and droplets positive for Signal β, such as the droplet indicated at **120**, are hatched according to fluorophore **94** (see FIG. **6**). Each double-positive droplet **170** is double-hatched and indicated by dashed box **164**.

The single-positive signatures indicated at **160** and **162** unambiguously identify corresponding droplets **118**, **120** as containing at least one copy of Target **1** or Target **2**, respectively, and no copy of Target **3**. Accordingly, the number of each type of single-positive droplet may be used, in a ratio with the total number of droplets, to calculate an average level of Target **1** and of Target **2**. However, this estimate may not be accurate enough if droplets contain multiple target molecules, because the estimate ignores any droplets containing Target **1** and/or Target **2**, but having the signature of Target **3**. These droplets can produce the same Target **3** signature while containing Targets **1+2**, Targets **1+3**, or Targets **2+3**. If the concentration of each target is low enough, the frequency of droplets containing at least two different targets may be negligible and/or ignored. Here, the concentrations of Targets **1**, **2**, and **3** are high enough to produce, on average, only about one droplet with both of Targets **1** and **2** per twenty droplets (see FIGS. **4** and **5**). Accordingly, one of double-positive droplets **170** is expected to contain both of Targets **1** and **2**, and the other two double-positive droplets are expected to contain Target **3**.

It is not necessary to know the target composition of each double-positive droplet. Instead, it is sufficient to know the frequency of droplets with each target signature. Poisson statistics then may be utilized to calculate the average level of each target. Calculation may be performed, in some cases by finding solutions to a series of simultaneous equations, such as numerically, to obtain a best-fit, or by a closed-form approach, among others.

## Example 2

### Exemplary Target-Specific Probes for Composite Signals

This example describes additional, exemplary target-specific probes that may be utilized in any suitable multiplexed digital assay; see FIGS. **9**A, **9**B, and **10**. The principles explained here may be extended to any number of signals and/or targets.

FIGS. **9**A, **9**B, and **10** shows third target **140** of FIG. **6** and other exemplary probe configurations of probes **180**, **182** (Probes **3**A and **3**B) each specific for Target **3** (and/or an amplicon thereof). Probes **3**A and **3**B may be used together, in place of Probe **3** of FIG. **6**, and in conjunction with Probe **1** and Probe **2** of FIG. **6**, to create only a pair of composite signals for Targets **1** to **3** in a digital amplification assay.

One of the probes (e.g., Probe **3**A) may include fluorophore **92** and the other probe (e.g., Probe **3**B) may include fluorophore **94**. Accordingly, light emitted by Probe **3**A can be detected in the same detection channel as light from Probe **1** of FIG. **6**, and light emitted by Probe **3**B in the same channel as Probe **2**.

FIGS. **9**A and **9**B show Probes **3**A and **3**B binding specifically to distinct sites of Target **3**. The probes may bind to non-overlapping (or only partially overlapping) sites on the same strand of the target (FIG. **9**A). Alternatively, the probes may bind to opposite strands of Target **3** (FIG. **9**B).

FIG. **10** shows Probes **3**A and **3**B that are capable of binding to the same site of Target **3** (and/or an amplicon thereof). Each of the probes may contain the same oligonucleotide **144** and thus differ only by the particular fluorophore (**92** or **94**) attached to oligonucleotide **144**. The probes of this example may be blended in any suitable ratio or set of different ratios for a multiplexed assay.

## Example 3

### Exemplary Tailed Primers and Shared Probes for Composite Signals

This example describes exemplary shared probes that enable creation of composite signals, and exemplary tailed primers forming binding sites for the shared probes; see FIG. **11**. The principles explained here may be extended to any suitable number of composite signals and/or targets.

FIG. **11** shows three targets **80**, **82**, and **140** (i.e., Targets **1** to **3** of FIG. **6**) and corresponding primers that enable assay of the three targets with only two probes, namely, probe **190** (Probe A) and probe **192** (Probe B), in a digital amplification assay. Probe A and Probe B may include respective fluorophores and a quencher (e.g., see FIG. **3**).

Target **1** may be amplified with a pair of forward and reverse primers **194**, **196**. Forward primer **194** may be a tailed primer with a 3' binding portion **198** that is complementary to a region of Target **1** and a 5' tail portion **200** that is not. The tail portion may introduce a binding site for Probe A into the resulting amplicon, indicated by a dashed line at **202**, such that Probe A (like Probe **1** of FIG. **6**) can report amplification of Target **1**.

Target **2** may be amplified with a pair of forward and reverse primers **204**, **206**. Reverse primer **206** may be structured analogously to forward primer **194** for Target **1**, with a 3' binding portion complementary to a region of Target **2** and a 5' tail portion **207** that is not. The tail portion may introduce a binding site for Probe B into the resulting amplicon, such that Probe B (like Probe **2** of FIG. **6**) can report amplification of Target **2**.

Target **3** may be amplified with a pair of forward and reverse primers **208**, **210**. Both of the primers may be structured analogously to forward primer **194**, with a 3' binding portion complementary to a region of Target **3** and a 5' tail portion that is not. Tail portions **200**, **207** of primers **208**, **210** may introduce respective binding sites for Probes A and B into the amplified product, such that a combination of both Probe A and Probe B (like Probe **3** of FIG. **6**) reports amplification of Target **3**.

## Example 4

### Exemplary Ligation Strategy to Enable Shared Probes

This example describes an exemplary ligation strategy to enable the use of shared probes; see FIG. **12**. The principles explained here may be extended to any number of composite signals and/or targets.

FIG. **12** shows Target **3** (at **140**) of FIG. **6** and another exemplary probe and primer configuration that enables assay of Targets **1** to **3** of FIG. **6** in a digital amplification assay with only Probes **1** and **2** (at **84** and **86**) of FIG. **6**. The ligation, extension, and digestion steps presented below may be performed in any suitable order and before or after a sample providing Target **3** is separated into partitions.

A template **220** (and/or a complementary strand and/or amplicon thereof) may be designed to bind each of Probes **1** and **2**.

Template **220** also may be designed to bind to adjacent regions of Target **3** via opposing end regions **222**, **224** of the template. (The template may be described as a molecular inversion "probe," but is generally not attached to a fluorophore.) The 5' and 3' termini of the template may be joinable directly to one another by ligation when bound to Target **3** or may form a gap **226** of one or more nucleotides between the aligned 5' and 3' termini of the template. The gap may be closed by extending the 3' terminus of the template, while bound to Target **3**, before ligation of the template to form a closed loop. After ligation, and optional extension, copies of the template that fail to ligate (and thus have not found a copy of Target **3** for binding), may be degraded by an exonuclease. Ligated copies of the template may be resistant to this degradation, such that the number of ligated template molecules corresponds to the number of Target **3** molecules.

Ligated template **220** (and/or a complementary strand thereof) may provide one or more sites for binding of at least one primer **228** (or **230**). The primer may amplify the ligated template by rolling circle amplification. Alternatively, or in addition, a pair of primers **228**, **230** (forward and reverse) may be included to produce a cascade of amplification. In some embodiments, ligated template **220** may be linearized by cleavage at a predefined site **232** before amplification with primers **228**, **230**. In any event, the presence of Target **3** in partitions is reported by a combination of both Probes **1** and **2** in this embodiment.

## Example 5

### Exemplary Shared Probes with Multi-target Specificity

This example describes exemplary shared probes each capable of binding to sequence regions of two different targets; see FIG. **13**. The principles explained here may be extended to any number of composite signals and/or targets.

FIG. **13** shows targets **80**, **82**, **140** (i.e., Targets **1** to **3**) bound by probes with multi-target specificity. In particular, a probe **240** (Probe **1**/**3**) includes an oligonucleotide **242** capable of binding to a sequence region present in Target **1** and another sequence region present in Target **3**. Also, another probe **244** (Probe **2**/**3**) includes an oligonucleotide **246** capable of binding to a sequence region present in Target **2** and another sequence region present in Target **3**.

## Example 6

### Increasing Multiplex Levels in Digital Amplification without Additional Detection Channels

This example describes an exemplary approach for increasing the multiplex level of a multiplexed digital amplification assay.

A. Introduction

The ability to measure multiple targets simultaneously (multiplexing) within every partition of a digital amplification system is often limited by the detection approach. Commonly one measures fluorescence to classify partitions as positive (if the measured fluorescence is high) or negative (if the measured fluorescence is low). Some chemistries, such as TaqMan, allow measurements of several targets simultaneously by utilizing target-specific probes labeled with different dyes. If the detector can measure the fluorescence

emitted by R different dyes, then the digital amplification system is effectively capable of measuring R different targets. Typically, instruments that are capable of detecting more colors are more expensive than those with fewer colors. Increasing the number of detectable dyes is expensive and is impractical beyond a certain number. On the other hand, many applications especially where sample is limited could benefit greatly from higher degrees of multiplexing.

This example presents an approach that, given an instrument capable of detecting multiple colors, can dramatically increase the number of simultaneously measured targets without requiring any changes to the detection optics of the instrument. The standard approach of designing assays is that a given target is assessed based on fluorescence produced from a single probe with a single dye. Thus, if the instrument is capable of detecting two colors, such as the light emitted from the dyes FAM and VIC, one measures the concentration of one target by counting the number of partitions with positive FAM signals and another target by counting the number of partitions with positive VIC signals.

One can design assays that produce fluorescence on multiple channels simultaneously. If processed on a digital amplification platform with a large number of partitions these assays can be multiplexed together with single channel assays and can be measured by counting the number of partitions with fluorescence on both channels.

B. Example with Two-color FAM-VIC Detection

Assuming we are looking at two unlinked loci (target **1** and target **2**), and given some number of FAM-only positive droplets as well as some number of VIC-only positive droplets, we can estimate how many FAM-VIC double-positive droplets we expect. If we are operating at low concentrations this number should be small and can be worked out in a straightforward fashion.

If we set up a third assay (target **3**) such that it has two additional probes—one labeled with FAM and one labeled with VIC, we can estimate the concentration of this third target locus by how many excess FAM-VIC double-positive droplets we have compared to the expectation. This would reduce the overall precision, but not much, and basically not at all if we are operating in a dilute regime (i.e., the total number of droplets is much larger than the number of positive droplets). Below is an example of an algorithm that can be used to determine the concentration of the excess FAM-VIC double positive droplets (or other partitions).

The use of multiple probes labeled with the same dye will increase the fluorescence of the negative droplets, which can present a challenge in extreme cases if fluorescence of the negative droplets starts approaching that of the positive droplets. This challenge can be addressed effectively by using sufficiently robust assays. One can also use common probes (e.g., see Examples 3-5) and avoid the elevation of negative fluorescence altogether. For the above example, we can consider using a common FAM probe for target **1** and target **3** and a common VIC probe for target **2** and target **3** by utilizing tailed primers or locked nucleic acid probes.

C. Additional Considerations

One gains an ever-larger advantage from this approach when one uses four or more colors. There are six combinations of two colors if one has four to choose from. Together with single colors, this would give a total of ten reporters. If we go further and use triplets of colors we would end up with 13 reporters.

The advantage of using this multi-color scheme becomes more pronounced with higher numbers of partitions. For that reason, this approach is of particular utility when combined with more recent implementations of digital amplification such as digital PCR in droplets where thousands or millions of partitions can be produced in an easy and cost effective manner.

Several assay schemes can be employed to assess a target with multiple colors simultaneously. One could design a multi-labeled probe—e.g., a single probe can be labeled with both FAM and VIC on the same molecule (e.g., see FIG. **6**). As another example, the same oligonucleotide may be labeled separately with FAM and VIC, to produce a FAM-labeled version and a VIC-labeled version of the same probe, and then the two versions mixed (e.g., see FIG. **9**B). In other cases, such as for a TaqMan assay, two probes can be designed to bind to different regions of the same amplicon strand (e.g., see FIG. **9**A). Alternatively, the probes can bind to opposite strands of the amplicon (e.g., see FIG. **10**), which may position the dyes away from the quenchers and facilitate the fluorescence increase from the bound probes.

This approach is general and can be used with a range of chemistries including ligation chain reaction, molecular beacons, scorpion probes, molecular inversion probes, or the like.

D. Mathematical Approach for Estimating Excess FAM-VIC Droplets

The following is an example of an algorithm that can be used to estimate concentrations of a joint FAM-VIC species (e.g., target **3** of FIGS. **6-8**).

1. Get 2×2 table of FAM versus VIC counts.
2. Compute concentration of distinct FAM and joint FAM-VIC as if there are 1 species.
3. Compute concentration of distinct VIC and joint FAM-VIC as if there are 1 species.
4. Try out different concentrations of joint FAM-VIC (from which the concentration of distinct FAM and distinct VIC can be found), and find the best fit of the probability table (Table 1) with the observed counts.

TABLE 1

| | FAM− | FAM+ |
|---|---|---|
| VIC+ | (1 − f) v (1 − c) | 1 − sum of others |
| VIC− | (1 − f) (1 − v) (1 − c) | f (1 − v) (1 − c) |

E. MATLAB Implementation of the Algorithm

Below is an example of a MATLAB implementation of the algorithm. Note that the algorithm can be expanded in a straightforward fashion to high order multiplexes.

```
% Consider three types of DNA fragments: Fam-Vic together,
% Fam fragment, Vic fragment. We observe some probabilities (counts in
% FAM-VIC cross plot), and the goal is to infer the concentrations.
% First let us do forward. Given concentrations, compute counts. Then to do
% inverse, we simply try out different values of concentrations and select
% one which gives actual counts.
N=20000;
A=10000;
B=20000;
AB=10000; % Joined together
cA=A/N;
cB=B/N;
cAB=AB/N;
fprintf(1, '% f % f % fn', cAB, cA, cB);
pA=1−exp(−cA);
pB=1−exp(−cB);
```

```
pAB=1−exp(−cAB);
% A is X and B is Y in cross plot
p(2,1)=(1−pA)*(1−pB)*(1−pAB); % Bottom left
p(2,2)=pA*(1−pB)*(1−pAB); % Bottom right
p(1,1)=(1−pA)*pB*(1−pAB); % Top Left
p(1,2)=1−p(2,1)−p(2,2)−p(1,1); % Top Right
disp(round(p*N));
% Also compute marginals directly
cAorAB=(A+AB)/N; %=c_A+c_AB;
cBorAB=(B+AB)/N; %=c_B+c_AB;
pAorAB=1−exp(−cAorAB); % Can be computed from p too
pBorAB=1−exp(−cBorAB);
% Inverse
H=p*N; % We are given some hits
% H=[08000; 20000];
% Compute prob
estN=sum(H(:));
i_p=H/estN;
i_pAorAB=i_p(1,2)+i_p(2,2);
i_pBorAB=i_p(1,1)+i_p(1,2);
i_cAorAB=−log(1−i_pAorAB);
i_cBorAB=−log(1−i_pBorAB);
maxVal=min(i_cAorAB, i_cBorAB);
delta=maxVal/1000;
errArr=[ ];
gcABArr=[ ];
forgcAB=0:delta:maxVal
gcA=i_cAorAB−gcAB;
gcB=i_cBorAB−gcAB;
gpA=1−exp(−gcA);
gpB=1−exp(−gcB);
gpAB=1−exp(−gcAB);
gp(2,1)=(1−gpA)*(1−gpB)*(1−gpAB); % Bottom left
gp(2,2)=gpA*(1−gpB)*(1−gpAB); % Bottom right
gp(1,1)=(1−gpA)*gpB*(1−gpAB); % Top Left
gp(1,2)=1−gp(2,1)−gp(2,2)−gp(1,1); % Top Right
gH=gp*estN;
err=sqrt(sum((H(:)−gH(:)).^2));
errArr=[errArr; err];
gcABArr=[gcABArr; gcAB];
end
figure, plot(gcABArr, errArr);
minidx=find(errArr==min(errArr(:)));
minidx=minidx(1);
estAB=gcABArr(minidx);
estA=i_cAorAB−estAB;
estB=i_cBorAB−estAB;
fprintf(1, '% f % f % f\n', estAB, estA, estB);
gpA=1−exp(−estA);
gpB=1−exp(−estB);
gpAB=1−exp(−estAB);
gp(2,1)=(1−gpA)*(1−gpB)*(1−gpAB); % Bottom left
gp(2,2)=gpA*(1−gpB)*(1−gpAB); % Bottom right
gp(1,1)=(1−gpA)*gpB*(1−gpAB); % Top Left
gp(1,2)=1−gp(2,1)−gp(2,2)−gp(1,1); % Top Right
gH=gp*estN;
disp(round(gH));
% Confirm the results using simulation
numMolA=round(estA*estN);
numMolB=round(estB*estN);
numMolAB=round(estAB*estN);
A=unique(randsample(estN, numMolA, 1));
B=unique(randsample(estN, numMolB, 1));
AB=unique(randsample(estN, numMolAB, 1));
U=1:estN;
notA=setdiff(U, A);
notB=setdiff(U, B);
notAB=setdiff(U, AB);
AorBorAB=union(A, union(B, AB));
none=setdiff(U, AorBorAB);
simcount(2,1)=length(none);
simcount(2,2)=length(intersect(A, intersect(notB, notAB)));
simcount(1,1)=length(intersect(B, intersect(notA, notAB)));
simcount(1,2)=length(AorBorAB)−simcount(2,2)−sim-
  count(1,1);
disp(simcount);
```

Example 7

Algorithm for Computation of DNA Fragmentation or for Digital Amplification Multiplexing

This example describes an exemplary algorithm to compute a level of DNA fragmentation and/or levels of target in a multiplexed amplification assay.

A. Introduction

1. Totally Fragmented Targets

Consider two DNA targets T**1** and T**2** corresponding to two dyes FAM and VIC, respectively. Let T**1** and T**2** be always on separate DNA fragments, as illustrated schematically in FIG. **14**. Let the number of DNA fragments with T**1** and T**2** targets be M**1** and M**2**, respectively.

Let the counts of FAM and VIC positive partitions be N**1** and N**2**, respectively. Note that N**1** and N**2** will be smaller than M**1** and M**2**, respectively, as there can be multiple DNA fragments in a partition. Let the total number of partitions be N. We will refer to digital amplification partitions simply as partitions. In this case, we can expect to see the counts of partitions listed in Table 2.

TABLE 2

| | VIC Negative | VIC positive | Total |
|---|---|---|---|
| FAM Positive | N1 · (N − N2)/N | N1 · N2/N | N1 |
| FAM Negative | (N − N1) · (N − N2)/N | (N − N1) · N2/N | N − N1 |
| Total | N − N2 | N2 | N |

If we denote the probability of seeing a partition to be FAM positive as p**1**=N**1**/N, and of seeing a partition to be VIC positive as p**2**=N**2**/N, then the probability table is given by Table **3**.

TABLE 3

| | VIC Negative | VIC positive | Probability |
|---|---|---|---|
| FAM Positive | p1 · (1 − p2) | p1 · p2 | p1 |
| FAM Negative | (1 − p1) · (1 − p2) | (1 − p1) · p2 | 1 − p1 |
| Probability | 1 − p2 | p2 | 1 |

In this case, we can say that 100% fragmentation exists.

We can compute the number of T**1** and T**2** molecules, M**1** and M**2**, respectively as follows, wherein (where log=$\log_e$):

$$M1=-N\log(1-p1)$$

$$M2=-N\log(1-p2)$$

(Given N digital partitions in which P are positive, the number of molecules is M=−N log(1−P/N).)

2. No Fragmentation

Now consider the other extreme. Both targets T**1** and T**2** are always found together on the same DNA fragments (e.g., see FIG. **15**). They are linked, perhaps because their loci are quite close to each other on the same part of a chromosome, and fragmentation of the chromosome during DNA isolation did

not separate the loci. Therefore, N1=N2. The expected counts and probabilities are listed in Tables 4 and 5, respectively.

TABLE 4

| | VIC Negative | VIC positive | Total |
|---|---|---|---|
| FAM Positive | 0 | N1 | N1 |
| FAM Negative | N − N1 | 0 | N − N1 |
| Total | N − N1 | N1 | N |

TABLE 5

| | VIC Negative | VIC positive | Probability |
|---|---|---|---|
| FAM Positive | 0 | p1 | p1 |
| FAM Negative | (1 − p1) | 0 | 1 − p1 |
| Probability | 1 − p1 | p1 | 1 |

In this case, we can say that 0% fragmentation exists.

We can compute the number of T1 and T2 molecules as follows, where p1=N1/N:

$$M1=-N\log(1-p1)$$

$$M2=-N\log(1-p1)$$

3. Partial Fragmentation

In the intermediate situation, where the targets are together on some fragments, but also happen to be on separate fragments, then we have partial fragmentation (e.g., see FIG. **16**).

Suppose we have M3 molecules of linked T1 and T2 fragments, M1 molecules of separate T1 fragments, and M2 molecules of separate T2 fragments.

We can make a table of counts of partitions (Table 6).

TABLE 6

| | VIC Negative | VIC positive | Total |
|---|---|---|---|
| FAM Positive | N01 | N11 | N1 |
| FAM Negative | N00 | N10 | N − N1 |
| Total | N − N2 | N2 | N |

4. Problem Statement

How can we find the number of molecules M1, M2 and M3, and thereby getting extent of fragmentation? For example if M1=M2=M3, then we would say that there is 50% fragmentation, as 50% of linked molecules got fragmented into separate fragments and 50% remained intact.

B. Multiplexing of PCR to Many Targets Using Few Colors

We have an algorithm that provides a solution to the above problem, and there is another interesting application for the algorithm. With this algorithm and by using a FAM probe for target T1, a VIC probe for target T2, and both FAM and VIC probes placed close to each other for target T3, we can achieve multiplexing of 3 targets by using 2 colors. Basically, we get a third color for "free" using the algorithm.

Now consider the case if there are 3 dyes. Thus, we will have a 2×2×2 table of 8 observed counts.

There are seven different kinds of targets: T1, T2, T3, T12, T23, T13, and T123. Here, for example, T12 means a target that is amplified to produce an amplicon bound by Dye 1 and Dye 2 probes, and T123 means a target that is amplified to produce an amplicon bound by one or more probes containing all three dyes, and likewise for the others.

If we have 4 dyes, then we have $2^4$=16 counts, and we can now multiplex quantitation of $2^4-1$=15 target genes. In general, with R colors, we have $2^R$ observed counts and we can have $2^R-1$ targets.

C. Solution for 2 Dyes and 3 Targets

Suppose we are given the counts listed in Table 7.

TABLE 7

| | VIC Negative | VIC positive | Total |
|---|---|---|---|
| FAM Positive | N01 | N11 | N1 |
| FAM Negative | N00 | N10 | N − N1 |
| Total | N − N2 | N2 | N |

Consider the following three cases:

1. We turn off VIC, as if VIC cannot be seen at all.
2. We turn off FAM, as if FAM cannot be seen at all.
3. We consider both FAM and VIC as if they are only one color.

We will have the following three observations:

1. Turning off VIC: We will be able to see T1 or T12, together and indistinguishably, as if there were one target. It gives the total number of molecules of T1 and T12, as if there were one target species.
2. Turning off FAM: We will be able to count T2 or T12, indistinguishably, as if there were one target. It gives the total number of molecules of T2 and T12, as if there were one target species.
3. Considering both FAM and VIC indistinguishably: We will be able to count T1, T2, or T12, indistinguishably. It gives the total number of molecules of T1, T2, and T12, as if there were one target species.

This allows us to step 3 equations in 3 unknowns. We can show these three cases in the form of a table.

TABLE 8

| Visible Dyes | Invisible Dyes | Distinct Dyes | Indistinct Dyes | Target Detected | Positive Count | Molecules |
|---|---|---|---|---|---|---|
| FAM | VIC | — | — | {T1, T12} | N1 | −log(1 − N1/N) |
| VIC | FAM | — | — | {T2, T12} | N2 | −log(1 − N2/N) |
| FAM, VIC | — | — | {FAM, VIC} | {T1, T2, T12} | N01 + N10 + N11 | −log(1 − (N01 + N10 + N11)/N) |

The three linear equations in three unknowns M1, M2 and M12 are:

$$\begin{bmatrix}101\\011\\111\end{bmatrix}\begin{bmatrix}M1\\M2\\M12\end{bmatrix}=\begin{bmatrix}M_{1\ or\ 12}\\M_{2\ or\ 12}\\M_{1\ or\ 2\ or\ 12}\end{bmatrix}=\begin{bmatrix}-N\log\left(1-\frac{N1}{N}\right)\\-N\log\left(1-\frac{N2}{N}\right)\\-N\log\left(1-\frac{N01+N10+N11}{N}\right)\end{bmatrix}$$

We solve the above equations to get values of M1, M2, and M12.

We can then compute the extent of fragmentation in % as follows:

$$M=(M1+M2)/2$$

$$F=M/(M+M12)*100$$

D. Algorithm Steps

Now we write down the steps of the algorithm clearly using the input of Table 9.

TABLE 9

| | VIC Negative | VIC positive | Total |
|---|---|---|---|
| FAM Positive | N11 | N12 | N1 |
| FAM Negative | N21 | N22 | N – N1 |
| Total | N – N2 | N2 | N |

Algorithm:

Step 1. Compute the three entities:

$$M_{1or12}=-N\cdot\log(1-N1/N)$$

$$M_{2or12}=-N\cdot\log(1-N2/N)$$

$$M_{1or2or12}=-N\cdot\log(1-(N01+N10+N11)/N)$$

Step 2. Solve the following linear equations:

$$\begin{bmatrix}101\\011\\111\end{bmatrix}\begin{bmatrix}M1\\M2\\M12\end{bmatrix}=\begin{bmatrix}M_{1\ or\ 12}\\M_{2\ or\ 12}\\M_{1\ or\ 2\ or\ 12}\end{bmatrix}=\begin{bmatrix}-N\log\left(1-\frac{N1}{N}\right)\\-N\log\left(1-\frac{N2}{N}\right)\\-N\log\left(1-\frac{N01+N10+N11}{N}\right)\end{bmatrix}$$

Step 3. Compute extent of fragmentation.

$$M=(M1+M2)/2$$

$$F=M/(M+M12)*100.$$

Step 4. Compute confidence intervals based on the concentration and expected number of positive counts. To compute confidence interval of F, we note that it is ratio of two random variables. Then we apply techniques to estimate confidence interval of ratio of two random variables for F.

The output is M**1**, M**2**, and F and their confidence intervals.

E. Outline of an Alternative Solution

Now we present an alternative solution to the problem of partial fragmentation phrased in terms of optimization of an objective criterion. First let us make the following table. Depending upon what type of molecules we have in a partition we will have corresponding FAM and VIC colors of the partition.

Table 10 maps molecules into partition colors.

TABLE 10

| Presence of Molecules in a Partition | | | Partition Color | |
|---|---|---|---|---|
| T1 | T2 | T12 | FAM (T1) | VIC (T2) |
| 0 | 0 | 0 | Neg | Neg |
| 0 | 1 | 0 | Neg | Pos |
| 1 | 0 | 0 | Pos | Neg |
| 0 | 0 | 1 | Pos | Pos |
| 0 | 1 | 1 | | |
| 1 | 0 | 1 | | |
| 1 | 1 | 0 | | |
| 1 | 1 | 1 | | |

Suppose we make a "guess" of M**1**, M**2**, and M**12**. We can compute the probabilities of a partition having a copy of the T**1**, T**2**, or T**12** target using inverse equations:

$$p12=1-\exp(-M12/N)$$

$$p1=1-\exp(-M1/N)$$

$$p2=1-\exp(-M2/N).$$

From these probabilities we can compute the predicted counts as shown in Table 11.

TABLE 11

| | VIC Negative | VIC positive |
|---|---|---|
| FAM Positive | p1(1 – p2)(1 – p12)N | (1 – (sum of other 3 cells in this table))N |
| FAM Negative | (1 – p1)(1 – p2)(1 – p12)N | (1 – p1)p2(1 – p12)N |

The probabilities above can be filled using Table 10, which maps presence of molecules into partition colors.

If our "guess" is really correct, then the predicted counts will "match" closely with our expected counts. Thus an optimization algorithm under the above objective criterion that needs to be minimized could also be used to solve the problem.

M**1**, M**2**, M**12**=best guess=least deviation of predicted counts and actual counts

We can then compute the extent of fragmentation in % as follows:

$$M=(M1+M2)/2$$

$$F=M/(M+M12)*100.$$

F. Solution for 3 Dyes and 7 Targets

To solve the problem for greater number of dyes, we need more equations as there are more unknowns. For 3 dyes and 7 targets, we need 7 equations to find the concentration of these 7 targets.

We have Table 12 with $2^3$=8 rows of counts of partitions depending upon which dyes are positive.

Denote the three dyes by D**1**, D**2** and D**3**.

TABLE 12

| Presence of color in a partition | | | |
|---|---|---|---|
| D1 | D2 | D3 | Count of Partitions |
| Neg | Neg | Neg | N000 |
| Neg | Pos | Neg | N010 |
| Pos | Neg | Neg | N100 |
| Neg | Neg | Pos | N001 |
| Neg | Pos | Pos | N011 |
| Pos | Neg | Pos | N101 |
| Pos | Pos | Neg | N110 |
| Pos | Pos | Pos | N111 |
| Total | | | N |

Consider Table 13.

TABLE 13

| Visible Dyes | Invisible Dyes | Distinct Dyes | Indistinct Dyes | Target Detected | Positive Count |
|---|---|---|---|---|---|
| D1, D2, D3 | — | — | {D1, D2, D3} | {T1, T2, T3, T12, T13, T23, T123} | C1 = N – N000 |

TABLE 13-continued

| Visible Dyes | Invisible Dyes | Distinct Dyes | Indistinct Dyes | Target Detected | Positive Count |
|---|---|---|---|---|---|
| D1 | D2, D3 | — | — | {T1, T12, T13, T123} | C2 = N100 + N101 + N110 + N111 |
| D2 | D1, D3 | — | — | {T2, T12, T23, T123} | C3 = N010 + N011 + N110 + N111 |
| D3 | D1, D2 | — | — | {T3, T13, T23, T123} | C4 = N001 + N011 + N101 + N111 |
| D1, D2 | D3 | D1, D2 | — | {T1, T13} | Compute concentration $M_{1 or 13}$ by solving 2 dye problem* |
| D1, D3 | D2 | D1, D3 | — | {T1, T12} | Compute concentration $M_{1 or 12}$ by solving 2 dye problem* |
| D2, D3 | D1 | D2, D3 | — | {T2, T12} | Compute concentration $M_{2 or 12}$ by solving 2 dye problem* |

*Note that for row 5, we could have also detected {T2, T23} or {T12, T123}, and for row 6, {T3, T23}, and for row 7, {T3, T13} and {T23, T123}. This choice does not matter and all lead to the same results.

We make 3 recursive calls, in rows 5, 6 and 7.

For example, in row 5, we are really solving 2 dyes, D**1** and D**2**, case with 3 targets: {T**1**, T**13**} as one target, {T**2**, T**23**} as second target, and {T**12**, T**123**} as third linked target.

Similarly, in rows 6 and 7, we make recursive calls to solve the simpler problem of 2 dyes and 3 targets.

The system of equation looks like as follows:

$$\begin{bmatrix} 1111111 \\ 1001101 \\ 0101011 \\ 0010111 \\ 1000100 \\ 1001000 \\ 0101000 \end{bmatrix} \begin{bmatrix} M1 \\ M2 \\ M3 \\ M12 \\ M13 \\ M23 \\ M123 \end{bmatrix} = \begin{bmatrix} M_{all} \\ M_{1 \text{ or } 12 \text{ or } 13 \text{ or } 123} \\ M_{2 \text{ or } 12 \text{ o } 23 \text{ or } 123} \\ M_{3 \text{ or } 13 \text{ or } 23 \text{ or } 123} \\ M_{1 \text{ or } 13} \\ M_{1 \text{ or } 12} \\ M_{2 \text{ or } 12} \end{bmatrix} = \begin{bmatrix} -N\log\left(1-\frac{C1}{N}\right) \\ -N\log\left(1-\frac{C2}{N}\right) \\ -N\log\left(1-\frac{C3}{N}\right) \\ -N\log\left(1-\frac{C4}{N}\right) \\ \text{Recursive call} \\ \text{Recursive call} \\ \text{Recursive call} \end{bmatrix}$$

The above system of linear equations can be solved to find concentrations of 7 targets.

G. R Dyes and $2^R-1$ Targets

Now we give the steps to generalize the algorithm to an arbitrary number of dyes.

This is a recursive algorithm. Since we have a solution for the case when R=2, we can solve for any number of R through recursion.

Input:

For R dyes, we are given a table of counts of partitions which has $2^R$ rows, for all possible combinations of presence or absence of colors in a partition.

Algorithm:

Step 1. Set up a system of $2^R-1$ linear equations. To obtain these equations consider different ways of making some colors invisible. Also consider the case when all colors are indistinguishable, which gives the first row. It can be shown that we can obtain $2^R-1$ equations in $2^R-1$ target concentrations (unknowns). When we make certain colors invisible, then we reduce the problem to a case with fewer colors, which can be solved recursively, all the way down to case when there are 2 dyes and 3 targets.

Step 2. Solve the equations.

Step 3. Compute confidence intervals based on the concentration and expected number of positive counts.

Output:

Concentrations of $2^R-1$ targets along with confidence intervals.

## Example 8

## Multi-Labeled Probes

This example describes exemplary multi-labeled probes and methods of using the probes in a multiplexed digital amplification assay; see FIGS. **17-22**. The multi-labeled probes may be utilized for the color-based multiplexed assays described in Section I and in Examples 6 and 7. Alternatively, or in addition, the multi-labeled probes may be utilized for intensity-based multiplexing, as described in U.S. Provisional Patent Application Ser. No. 61/507,082, filed Jul. 12, 2011; and U.S. Provisional Patent Application Ser. No. 61/510,013, filed Jul. 20, 2011, which are incorporated herein by reference.

Typical 5' nuclease assay (TaqMan) probes have a single fluorophore and a quencher, or two fluors where the second fluor acts to quench the first fluorophore through FRET (e.g., a TAMRA "quencher"). The fluorophore and quencher are typically attached to the 5'- and 3'-most bases/nucleotides of the oligonucleotide probe.

Oligonucleotide synthesis chemistry allows fluorophores to be added to internal nucleotides/bases of a probe. Attaching multiple fluorophores to one oligonucleotide probe allows creation of a wider range of probes, which can be used to enhance multiplexing capabilities. The ability to put multiple fluors on one probe allows the resulting emission fluorescence to be "tuned" to achieve more fluorescence signatures than are possible through single fluorophores. Probe spectra will generally be a composite result of the multiple fluorophores included. For fluorophores that can resonate or otherwise interact, the proximity of the fluorophores and location (internal or end label) will allow additional tuning possibilities. It is not necessary that the multiple fluorophores (or quenchers) be different. Addition of multiple molecules of the same fluor also may allow different fluorescence output on a per-probe basis. For some applications, it may be beneficial to put the quencher on the 5'-end of the oligonucleotide so that probe degradation removes the quencher, but leaves the other fluorophores attached on the remaining portion of the oligonucleotide. That way, when the quencher is cleaved off, the same signal is obtained from the probe, but each probe could have a different signature (e.g., probe **1**—FAM; probe **2**—FAM+Cy3; probe **3**—FAM+HEX; probe **4**—FAM+FAM; etc.).

The approach is also applicable to multiplexing for other target molecules. Although detection of target nucleic acids is clearly an area of great interest, it is also possible to apply this concept to other types of probes (for example, antibody probes for protein targets).

FIG. **17** show a schematic representation of an unlabeled oligonucleotide (A) and a set of exemplary multi-labeled probes (B-G) containing the oligonucleotide. Each solid circle and open circle represents a quencher (also termed a

quencher moiety) and a fluorophore, respectively, attached to the oligonucleotide. F**1** to F**4** identify structurally distinct fluorophores. The oligonucleotide may have any suitable length, such as 5 to 500, 10 to 200, or 15 to 100 nucleotides, among others.

The multi-labeled probes of FIG. **17** are as follows: B) oligonucleotide with a 5' quencher and fluorophores (F**1** and F**2**) at respective internal and 3'-end positions; C) oligonucleotide with a 5' quencher moiety and fluorophores (F**1** to F**4**) at 3' and multiple internal positions; D) oligonucleotide with a 5' quencher moiety and fluorophores (3×F**1**, F**2**) at 3' and multiple internal positions; E) oligonucleotide with a 5' quencher moiety and an internal quencher moiety plus fluorophores at 3' and internal positions; F) oligonucleotide with a 5' quencher moiety and fluorophores at 3' and internal positions, with greater separation between fluorophores; and G) oligonucleotide with fluorophores at the 5'-end and an internal position and a quencher at the 3'-end position.

FIG. **18** depicts separation of the quencher and fluorophores of a probe molecule during target amplification. A template molecule is shown being copied (complementarily) by DNA polymerase in the presence of an exemplary multi-labeled probe molecule (FIG. **17**B). A) Polymerase is extending a nucleic acid strand upstream of a probe binding site. B) **5'** nuclease activity of the polymerase removes the 5'-most nucleotides (including the quencher in this example) from the probe, allowing the quencher and fluorophores to be spatially separated. C) Polymerase continues to remove 5' nucleotides from the probe until binding of the probe becomes unstable and the remaining probe fragment dissociates from the template strand. Multiple fluorophores still may be connected to each other in the probe fragment, but are separated from the quencher.

FIG. **19** shows an exemplary two-dimensional histogram of droplet intensities, showing droplet clusters that may be obtained in a multiplexed digital amplification assay for three targets performed with a combination of single-labeled and dual-labeled probes each labeled with FAM, VIC, or both FAM and VIC. Here, the droplet cluster produced by target-3 positives is not resolved from droplets double-positive for both target **1** and **2**.

FIG. **20** shows another exemplary two-dimensional histogram of droplet intensities that may be obtained in the assay of FIG. **19**, with partial resolution of a cluster for target-1+2-positive droplets from a cluster for target-3-positive droplets.

Detector optics, particularly excitation sources and optical filters, may be selected to optimize the separation of clusters. For example, two clusters might be substantially overlapping (hard to separate) at a first wavelength condition but substantially non-overlapping at a second wavelength condition. Detectors with spectrophotometer gratings or exchangeable filter sets could provide greater flexibility in wavelength selection.

FIG. **21** shows an exemplary two-dimensional histogram of droplet intensities, illustrating two clusters of data points that may be obtained in a digital amplification assay performed with only a multi-labeled probe.

FIG. **22** shows another exemplary two-dimensional histogram of droplet intensities, illustrating various clusters of data points that may be obtained in a digital amplification assay performed as in FIG. **21**, but with the assay supplemented with a pair of single-labeled probes (for measuring target **1** and target **2** amplification), in addition to the multi-labeled probe (for measuring target **3** amplification). Putting multiple fluors on the same oligonucleotide can be more advantageous when combined with different levels of probe and/or with different fluors that have different spectral overlap in two (or more) detection channels. The example of FIG. **22** shows the dual fluor probe having slightly higher fluorescence than the FAM-only and VIC-only probes, but it could be either higher or lower (or the same). If about the same (or not), the color-based multiplexing approach disclosed herein may be utilized advantageously to determine average target levels.

Several chemistries can yield the multi-labeled probes disclosed herein, including labeled deoxynucleotides, click chemistry, and various other linker chemistries. The probes could be produced through custom synthesis by an oligonucleotide supplier. References describing exemplary synthetic routes for internal fluorophore incorporation are listed below and are incorporated herein by reference.

Haralambidis J., Chai M. and Tregear G. W. (1987) Preparation of base-modified nucleosides suitable for non-radioactive label attachment and their incorporation into synthetic oligodeoxyribonucleotides. Nucleic Acids Res. 15, 4857-4876.

Randolph J. B., and Waggoner A. S. (1997) Stability, specificity and fluorescence brightness of multiply-labelled fluorescence DNA probes. Nucleic Acids Research; 25:2923-2929.

Brumbaugh J. A., Middendorf L. R., Grone D. L., and Ruth J. L. Proc. Natl. Acad. Sci. USA 1988; 85:5610-5614.

Singh D., Vijayanti K., Ganesh K. N. (1990) Oligonucleotides, part 5+: synthesis and fluorescence studies of DNA oligomers d(AT) 5 containing adenines covalently linked at C-8 with dansyl fluorophore. Nucleic Acids Res.; 18:3339-3345.

Tae Seok Seo, Zengmin Li, Hameer Ruparel, and Jingyue Ju (2003) Click Chemistry to Construct Fluorescent Oligonucleotides for DNA Sequencing. J. Org. Chem.; 68: 609-612.

## Example 9

### Selected Embodiments

This example describes selected aspects and embodiments related to digital assays with combinatorial use of signals, presented without limitation as a series of numbered paragraphs. Each of these paragraphs can be combined with one or more other paragraphs, and/or with disclosure from elsewhere in the present disclosure, in any suitable manner. Some of the paragraphs below expressly refer to and further limit other paragraphs, providing without limitation examples of some of the suitable combinations.

A1. A method of performing a digital assay, comprising: (a) creating R signals representative of light detected from each of a plurality of partitions of a sample; and (b) estimating a concentration of more than R different targets in the partitions based on how the R signals vary relative to one another among the partitions.

B1. A method of performing a digital assay for more than R targets, comprising: (a) separating a sample into partitions; (b) creating R signals representative of light detected from the partitions; and (c) estimating a concentration of more than R targets in the partitions based on the R signals.

B2. The method of paragraph B1, wherein at least one of the R signals reports the presence or absence of a target in each partition independently of every other one of the R signals.

B3. The method of paragraph B1 or B2, wherein each of the R signals reports the presence or absence of a different target in each partition independently of every other one of the R signals.

B4. The method of any of paragraphs B1 to B3, wherein a combination of two or more of the R signals collectively reports the presence or absence an R+1 target in each partition.

B5. The method of any of paragraphs B1 to B4, further comprising a step of determining a number of the partitions that are positive for each of the R signals alone and a number that are positive for at least one combination of two or more of the R signals.

B6. The method of paragraph B5, wherein the step of estimating a concentration includes a step of estimating concentrations of each of the more than R targets that collectively correspond to the determined numbers of positives, if each target has a Poisson distribution among the partitions.

B7. The method of any of paragraphs B1 to B6, wherein the step of estimating includes a step of finding solutions to a set of simultaneous equations, and wherein the equations each have the same variables.

B8. The method of paragraph B7, wherein the simultaneous equations are linear equations.

B9. The method of paragraph B7 or B8, wherein the solutions are obtained by numerical analysis.

B10. The method of any of paragraphs B7 to B9, wherein the step of finding solutions includes a step of finding solutions to at least $2^R-1$ equations.

B11. The method of any of paragraphs B7 to B10, wherein each equation is based on copies of each target having a Poisson distribution among the partitions.

B12. The method of any of paragraphs B1 to B11, wherein each of the R signals is a composite signal that includes two or more integral signal portions corresponding to the presence or absence of different targets in individual partitions.

B13. The method of any of paragraphs B1 to B12, wherein the step of separating a sample forms the partitions with an average concentration per partition of less than about one copy of each of the more than R targets.

B14. The method of any of paragraphs B1 to B13, wherein the step of separating a sample forms one or more partitions containing no copies of a target for each of the more than R targets.

B15. The method of any of paragraphs B1 to B14, wherein the partitions are droplets.

B16. The method of any of paragraphs B1 to B15, wherein each of the R signals is representative of fluorescence emission that is detected.

B17. The method of any of paragraphs B1 to B16, further comprising a step of performing an amplification reaction in one or more of the partitions before the step of creating R signals.

B18. The method of paragraph B17, wherein the step of performing an amplification reaction includes a step of thermally cycling partitions.

B19. The method of any of paragraphs B1 to B18, wherein each target is a nucleic acid.

B20. The method of any of paragraphs B1 to B19, wherein the partitions include a first probe reporting a presence or absence of a first target molecule in individual partitions, a second probe reporting a presence or absence of a second target molecule in individual partitions, and at least one third probe reporting a presence or absence of a third target molecule in individual partitions.

B21. The method of claim B20, wherein the at least one third probe is a single third probe.

B22. The method of any of paragraphs B1 to B19, wherein the partitions include a first probe reporting a presence or absence of a first target molecule in individual partitions, a second probe reporting a presence or absence of a second target molecule in individual partitions, and wherein the first and second probes collectively report a presence or absence of a third target molecule in individual partitions.

B23. The method of any of paragraphs B1 to B22, wherein each of the R signals is representative at least predominantly of light detected from a different fluorophore.

B24. The method of any of paragraphs B1 to B23, wherein the partitions all have substantially same volume.

B25. The method of any of paragraphs B1 to B24, wherein at least one of the more than R targets is a linked version of at least two of the other R targets.

C1. A method of performing a digital assay, comprising: (a) separating a sample into partitions, with each partition capable of amplifying more than R targets, if present in the partition; (b) creating R signals representative of light detected from the partitions, wherein, for at least one of the more than R targets, amplification in a partition of the at least one target selectively changes only one of the R signals, and wherein, for at least one other of the more than R targets, amplification in a partition of at least one other target coordinately changes two or more of the signals; and (c) estimating a concentration of each of the more than R targets based on the R signals created.

C2. The method of paragraph C1, wherein the step of amplifying includes a step of thermally cycling the partitions before the step of creating.

C3. The method of paragraph C1 or C2, wherein the R signals are representative of light detected from fluid carrying droplets through an examination region.

C4. The method of any of paragraphs C1 to C3, wherein the step of creating includes a step of creating two or more signals that represent different wavelengths and/or wavelength ranges of detected light.

C5. The method of any of paragraphs C1 to C4, wherein the step of creating includes a step of creating two or more signals that are representative of a same wavelength range of detected light produced by illumination with a different wavelength or wavelength range for each of the two or more signals.

D1. A method of performing a multiplexed digital amplification assay, the method comprising: (a) amplifying more than R targets in partitions; (b) creating R signals representative of light detected in R different wavelength regimes from the partitions, where $R \geq 2$; and (c) calculating an average level of each target in the partitions based on the R signals, wherein the level calculated accounts for a coincidence, if any, of different targets in the same individual partitions. For example, if T denotes the number of targets, then T>R.

D2. The method of paragraph D1, wherein amplification of each target is reported by a different signal or combination of the signals than any of the other targets individually.

D3. The method of paragraph D1 or D2, wherein each of the signals reports amplification of a different combination of at least two of the targets.

D4. The method of paragraph D3, wherein the partitions are droplets, further comprising a step of determining a number of droplets exhibiting amplification of any of the at least two targets for each signal, and wherein the step of calculating is based on the number determined for each of the R signals.

D5. The method of any of paragraphs D1 to D4, wherein the step of calculating includes a step of finding solutions to a set of simultaneous equations.

D6. The method of paragraph D5, wherein there are T targets, wherein the step of finding solutions includes a step of finding solutions to T simultaneous equations, and wherein $R<T\leq 2^R-1$.

D7. The method of paragraph D5 or D6, wherein the solutions are obtained by numerical analysis.

D8. The method of any of paragraphs D1 to D7, wherein there are three targets, and wherein the signals representative of light are detected from two fluorophores associated with probes that bind to amplicons of respective targets during amplification.

D9. The method of paragraph D8, wherein the two fluorophores are VIC and FAM.

D10. The method of paragraph D8 or D9, wherein the partitions contain a first probe for a first of the three targets, a second probe for a second of the three targets, and a third probe for a third of the three targets, and wherein the first probe is labeled exclusively with VIC, the second probe is labeled exclusively with FAM, and the third probe is labeled with both VIC and FAM.

D11. The method of paragraph D10, wherein the third probe includes a FAM-labeled probe that is not labeled with VIC and a VIC-labeled probe that is not labeled with FAM.

D12. The method of any of paragraphs D1 to D11, wherein the average level is a concentration.

D13. The method of any of paragraphs D1 to D12, wherein the step of calculating an average level includes a step of determining a total number of amplification-positive partitions for each type of target and a step of determining a total number of partitions.

D14. The method of any of paragraphs D1 to D13, further comprising a step of distributing copies of the more than R targets among the partitions such that some partitions contain more than one copy of a given target.

D15. The method of any of paragraphs D1 to D13, wherein there are at least four targets.

E1. A method of performing a multiplexed digital amplification assay, the method comprising: (a) amplifying more than R targets in droplets; (b) creating R signals representative of light detected in R different wavelength regimes from the droplets, where $R \geq 2$; and (c) calculating an average level of each of the more than R targets by finding solutions to a series of simultaneous equations. For example, if T denotes the number of targets, then T>R.

E2. The method of paragraph E1, wherein amplification of each target is reported by a different signal or combination of the signals than any of the other targets individually.

E3. The method of paragraph E1 or E2, wherein each of the signals reports amplification of a different combination of at least two of the targets.

E4. The method of paragraph E3, further comprising a step of determining a total number of droplets that are amplification-positive for any of the at least two targets reported on by each signal, and wherein the step of calculating is based on the total number determined for each of the R signals.

E5. The method of any of paragraphs E1 to E4, wherein there are T targets, wherein the step of calculating includes a step of finding solutions to T simultaneous equations, and wherein $R<T \leq 2^R-1$.

E6. The method of any of paragraphs E1 to E5, wherein the solutions are obtained by numerical analysis.

E7. The method of any of paragraphs E1 to E6, wherein the level accounts for any coincidence of different targets in the same individual droplets.

F1. A method of performing a multiplexed digital amplification assay, the method comprising: (a) amplifying more than R targets in droplets; (b) creating R signals representative of light detected in R different wavelength regimes from the droplets, wherein $R \geq 2$ and each of the signals reports amplification of a different combination of at least two of the targets; and (c) calculating an average level of each target in the droplets based on the R signals and without determining which of the at least two targets for each signal amplified in individual amplification-positive droplets for such signal. For example, if T denotes the number of targets, then T>R.

F2. The method of paragraph F1, further comprising a step of determining a total number of droplets that are amplification-positive for any of the at least two targets reported on by each signal, and wherein the step of calculating is based on the total number determined for each of the R signals.

F3. The method of paragraph F1 or F2, wherein the step of calculating includes a step of finding solutions to a set of simultaneous equations.

G1. A composition, comprising: a droplet containing a probe, the probe including an oligonucleotide, a first fluorophore, a second fluorophore, and an energy transfer moiety, wherein the energy transfer moiety is a quencher and/or an energy transfer partner for one or both of the first and second fluorophores.

G2. The composition of paragraph G1, wherein the first fluorophore, the second fluorophore, and the energy transfer moiety are each covalently attached to the oligonucleotide.

G3. The composition of paragraph G1 or G2, further comprising a plurality of droplets containing the probe and disposed in a carrier fluid.

G4. The composition of any of paragraphs G1 to G3, wherein the droplet contains a template molecule and amplification reagents capable of amplifying at least a region of the template molecule, and wherein the probe is capable of binding to amplicons generated by amplification of the region of the template molecule.

G5. The composition of any of paragraphs G1 to G4, wherein the probe is a first probe, and wherein the droplet further comprises a second probe including the first fluorophore or the second fluorophore, but not both the first fluorophore and the second fluorophore.

G6. The composition of any of paragraphs G1 to G5, wherein the energy transfer moiety is a quencher attached to a nucleotide at the 5'-end of the oligonucleotide.

G7. The composition of any of paragraphs G1 to G6, wherein each of the first fluorophore, the second fluorophore, and the energy transfer moiety is attached to a different nucleotide of the oligonucleotide.

G8. The composition of paragraph G7, wherein a pair of the first fluorophore, the second fluorophore, and the energy transfer moiety are attached to the same nucleotide of the oligonucleotide.

G9. The composition of any of paragraphs G1 to G8, wherein at least one of the first fluorophore, the second fluorophore, and the energy transfer moiety is attached to a nucleotide of the oligonucleotide via another of the first fluorophore, the second fluorophore, and the energy transfer moiety.

G10. The composition of any of paragraphs G1 to G9, wherein a fluorophore or a quencher is attached to the 5'-end of the oligonucleotide, wherein a fluorophore or a quencher is attached to the 3'-end of the oligonucleotide, and wherein a fluorophore or a quencher is attached to a nucleotide intermediate the 5'-end and the 3'-end.

G11. The composition of any of paragraphs G1 to G10, wherein one or more of the first fluorophore, the second fluorophore, and the energy transfer moiety are attached to one or more internal nucleotides disposed intermediate the 5'-end and the 3'-end of the oligonucleotide.

The disclosure set forth above may encompass multiple distinct inventions with independent utility. Although each of these inventions has been disclosed in its preferred form(s), the specific embodiments thereof as disclosed and illustrated herein are not to be considered in a limiting sense, because numerous variations are possible. The subject matter of the

inventions includes all novel and nonobvious combinations and subcombinations of the various elements, features, functions, and/or properties disclosed herein. The following claims particularly point out certain combinations and subcombinations regarded as novel and nonobvious. Inventions embodied in other combinations and subcombinations of features, functions, elements, and/or properties may be claimed in applications claiming priority from this or a related application. Such claims, whether directed to a different invention or to the same invention, and whether broader, narrower, equal, or different in scope to the original claims, also are regarded as included within the subject matter of the inventions of the present disclosure. Further, ordinal indicators, such as first, second, or third, for identified elements are used to distinguish between the elements, and do not indicate a particular position or order of such elements, unless otherwise specifically stated.

We claim:

**1**. A method of performing a multiplexed digital amplification assay, the method comprising:

amplifying more than R targets in partitions;

creating R signals representative of light detected in R different wavelength regimes from the partitions, where R≥2; and

calculating an average level of each target in the partitions based on the R signals, wherein the level calculated accounts for a coincidence of all possible combinations of the more than R targets in the same individual partitions;

wherein the more than R targets include three targets, and wherein the average levels of the three targets are calculated based on light detected from only two fluorophores associated with probes that bind to amplicons of the three targets during amplification.

**2**. The method of claim **1**, wherein amplification of each target of the more than R targets is reported by a different signal or combination of the signals than any of the other more than R targets individually.

**3**. The method of claim **1**, wherein each of the signals reports amplification of a different combination of at least two of the more than R targets.

**4**. The method of claim **3**, wherein the partitions are droplets, further comprising a step of determining a number of droplets exhibiting amplification of any of the at least two targets for each signal, and wherein the step of calculating is based on the number determined for each of the R signals.

**5**. The method of claim **1**, wherein the two fluorophores are VIC and FAM.

**6**. The method of claim **5**, wherein the partitions contain a first probe for a first of the three targets, a second probe for a second of the three targets, and a third probe for a third of the three targets, and wherein the first probe is labeled exclusively with VIC, the second probe is labeled exclusively with FAM, and the third probe is labeled with both VIC and FAM.

**7**. The method of claim **6**, wherein the third probe includes a FAM-labeled probe that is not labeled with VIC and a VIC-labeled probe that is not labeled with FAM.

**8**. The method of claim **1**, wherein the average level is a concentration.

**9**. The method of claim **1**, wherein the step of calculating an average level includes a step of determining a total number of amplification-positive partitions for each type of the more than R targets and a step of determining a total number of partitions.

**10**. The method of claim **1**, further comprising a step of distributing copies of the more than R targets among the partitions such that some partitions contain more than one copy of a given target.

**11**. The method of claim **1**, wherein there are at least four targets.

**12**. A method of performing a multiplexed digital amplification assay, the method comprising:

amplifying targets in partitions;

detecting light from the partitions; and

calculating levels of at least three of the targets based on light detected from only two fluorophores, wherein the levels calculated account for a coincidence of all possible combinations of the at least three targets in the same individual partitions.

**13**. The method of claim **12**, wherein the partitions contain a first probe for a first target of the at least three targets, a second probe for a second target of the at least three targets, and a third probe for a third target of the at least three targets, and wherein the first probe is labeled exclusively with a first fluorophore, the second probe is labeled exclusively with a second fluorophore, and the third probe is labeled with the first fluorophore and the second fluorophore.

**14**. The method of claim **13**, wherein the third probe includes a probe labeled with the first fluorophore and not the second fluorophore and another probe labeled with the second fluorophore and not the first fluorophore.

**15**. The method of claim **13**, wherein the first fluorophore is VIC and the second fluorophore is FAM.

* * * * *

# EXHIBIT B

US009921154B2

(12) **United States Patent**
**Jouvenot et al.**

(10) **Patent No.: US 9,921,154 B2**
(45) **Date of Patent: Mar. 20, 2018**

(54) **MULTIPLEXED DIGITAL ASSAYS**

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( * ) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 889 days.

(21) Appl. No.: **14/099,750**

(22) Filed: **Dec. 6, 2013**

(65) **Prior Publication Data**

US 2014/0171341 A1 Jun. 19, 2014
US 2017/0205345 A9 Jul. 20, 2017

**Related U.S. Application Data**

(63) Continuation-in-part of application No. 13/424,304, filed on Mar. 19, 2012, now Pat. No. 9,222,128, and a continuation-in-part of application No. 13/548,062, filed on Jul. 12, 2012, now Pat. No. 8,951,939.

(60) Provisional application No. 61/454,373, filed on Mar. 18, 2011, provisional application No. 61/507,082, filed on Jul. 12, 2011, provisional application No. 61/510,013, filed on Jul. 20, 2011, provisional application No. 61/734,296, filed on Dec. 6, 2012.

(51) **Int. Cl.**

| | |
|---|---|
| ***G01N 33/48*** | (2006.01) |
| ***G01N 33/50*** | (2006.01) |
| ***G01N 21/64*** | (2006.01) |
| ***G06F 19/18*** | (2011.01) |
| ***G06F 19/12*** | (2011.01) |

(52) **U.S. Cl.**
CPC ..... ***G01N 21/64*** (2013.01); ***G06F 19/12*** (2013.01); ***G06F 19/18*** (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

| | | | | |
|---|---|---|---|---|
| 5,304,810 | A * | 4/1994 | Amos | 250/458.1 |
| 6,020,141 | A | 2/2000 | Pantoliano et al. | |
| 7,801,226 | B2 | 9/2010 | Suh et al. | |
| 8,148,515 | B1 | 4/2012 | Mao et al. | |
| 8,951,939 | B2 | 2/2015 | Saxonov et al. | |
| 9,156,010 | B2 | 10/2015 | Colston, Jr. et al. | |
| 9,222,128 | B2 | 12/2015 | Saxonov et al. | |

(Continued)

FOREIGN PATENT DOCUMENTS

| | | |
|---|---|---|
| AU | 2012231098 | 1/2017 |
| WO | 03064691 A2 | 8/2003 |

(Continued)

OTHER PUBLICATIONS

Ottesen et al. Microfluidic digital PCR enables multigene analysis of individual environmental bacteria. Science, 2006, vol. 314, pp. 1464-1467.*

(Continued)

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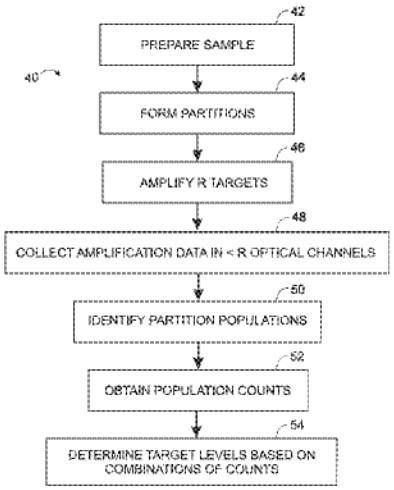
(57) **ABSTRACT**

System, including methods and apparatus, for performing a multiplexed digital assay.

**28 Claims, 15 Drawing Sheets**



(56) **References Cited**

U.S. PATENT DOCUMENTS

| | | | | |
|---|---|---|---|---|
| 9,702,822 | B2 | 7/2017 | Litterst et al. | |
| 2005/0266448 | A1 | 12/2005 | Hagiwara et al. | |
| 2006/0078888 | A1 | 4/2006 | Griffiths et al. | |
| 2009/0069194 | A1 | 3/2009 | Ramakrishnan | |
| 2009/0239308 | A1 | 9/2009 | Dube et al. | |
| 2009/0311713 | A1 | 12/2009 | Pollack et al. | |
| 2010/0022414 | A1 | 1/2010 | Link et al. | |
| 2010/0092973 | A1 | 4/2010 | Davies et al. | |
| 2010/0173394 | A1 | 7/2010 | Colston, Jr. et al. | |
| 2010/0233686 | A1 | 9/2010 | Higuchi et al. | |
| 2010/0248385 | A1 | 9/2010 | Tan et al. | |
| 2010/0304978 | A1 | 12/2010 | Deng et al. | |
| 2011/0000560 | A1 | 1/2011 | Miller et al. | |
| 2011/0104686 | A1 | 5/2011 | Litterst et al. | |
| 2011/0159499 | A1 | 6/2011 | Hindson et al. | |
| 2011/0244455 | A1 | 10/2011 | Larson et al. | |
| 2011/0250597 | A1 | 10/2011 | Larson et al. | |
| 2012/0122714 | A1 | 5/2012 | Samuels et al. | |
| 2012/0164690 | A1 | 6/2012 | Wang | |
| 2012/0208241 | A1 | 8/2012 | Link | |
| 2012/0219947 | A1 | 8/2012 | Yurkovetsky et al. | |
| 2012/0220494 | A1 | 8/2012 | Samuels et al. | |
| 2012/0252015 | A1 | 10/2012 | Hindson et al. | |
| 2012/0264646 | A1 | 10/2012 | Link et al. | |
| 2012/0302448 | A1 | 11/2012 | Hutchison et al. | |
| 2012/0309002 | A1 | 12/2012 | Link | |
| 2012/0316074 | A1 | 12/2012 | Saxonov | |
| 2012/0322058 | A1 | 12/2012 | Regan et al. | |
| 2012/0329664 | A1* | 12/2012 | Saxonov et al. | 506/9 |
| 2013/0017968 | A1 | 1/2013 | Gurtner et al. | |
| 2013/0040841 | A1 | 2/2013 | Saxonov et al. | |
| 2013/0059754 | A1 | 3/2013 | Tzonev | |
| 2013/0178378 | A1 | 7/2013 | Hatch et al. | |
| 2014/0057273 | A1 | 2/2014 | Litterst et al. | |

FOREIGN PATENT DOCUMENTS

| | | | |
|---|---|---|---|
| WO | 2006002167 | A2 | 1/2006 |
| WO | 2010036352 | A1 | 4/2010 |
| WO | 2011100604 | A2 | 8/2011 |
| WO | 2011143478 | A2 | 11/2011 |
| WO | 2012129187 | A1 | 9/2012 |
| WO | 2014031908 | A1 | 2/2014 |

OTHER PUBLICATIONS

Vogelstein et al. Digital PCR. PNAS, vol. 96, 1999, pp. 9236-9241.*

Arya, Manit et al., "Basic principles of real-time quantitative PCR", Expert Review of Molecular Diagnostics, vol. 5, No. 2, 2005, pp. 209-219.

Beer, N. Reginald et al., "On-Chip, Real-Time, Single-Copy Polymerase Chain Reaction in Picoliter Droplets", Analytical Chemistry, vol. 79, No. 22, Nov. 15, 2007, pp. 8471-8475.

Bhagwat, Arvind A., "Simultaneous detection of *Escherichia coli* O157:H7, Listeria monocytogenes and Salmonella strains by real-time PCR", International Journal of Food Microbiology, vol. 84, 2003, pp. 217-224.

Butler, John M. et al., "Capillary electrophoresis as a tool for optimization of multiplex PCR reactions", Fresenius Journal Analytical Chemistry, vol. 369, 2001, pp. 200-205.

Cawthon, Richard M., "Telomere measurement by quantitative PCR", Nucleic Acids Research, vol. 30, No. 10, 2002, pp. 1-6.

Dube, Simant et al., "Mathematical Analysis of Copy Number Variation in a DNA Sample Using Digital PCR on a Nanofluidic Device", PLoS ONE, vol. 3, Issue 8, Aug. 2008, pp. 1-9.

Higuchi, Russell et al., "Simultaneous Amplification and Detection of Specific DNA Sequences", Biotechnology, vol. 10, Apr. 1992 pp. 1-5.

Higuchi, Russell et al., "Kinetic PCR Analysis: Real-time Monitoring of DNA Amplification Reactions", Biotechnology, vol. 11, Sep. 1993, pp. 1-5.

Hindson, Benjamin J. et al, "High-Throughput Droplet Digital PCR System for Absolute Quantitation of DNA Copy Number", Analytical Chemistry, vol. 83, 2011, pp. 8604-8610.

Hua, Zhishan et al., "Multiplexed Real-Time Polymerase Chain Reaction on a Digital Microfluidic Platform", Analytical Chemistry, vol. 82, No. 6, Mar. 15, 2010, pp. 2310-2316.

Lind, Kristina et al., "Combining sequence-specific probes and DNA binding dyes in real-time PCR for specific nucleic acid quantification and melting curve analysis", BioTechniques. vol. 40, No. 3, Mar. 2006, pp. 315-318.

Mao, Fei et al., "Characterization of EvaGreen and the implification of its physicochemical properties for qPCR applications", BMC Biotechnology, vol. 7, No. 76, Nov. 9, 2007, pp. 1-16.

Markey, Amelia L. et al., "High-throughput droplet PCR", Methods, vol. 50, Feb. 2, 2010, pp. 277-281.

Martin, Kendall J. et al., "Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts", BMC Microbiology, vol. 5, No. 28, May 18, 2005, pp. 1-11.

McDermott, Geoffrey P. et al., "Multiplexed Target Detection Using DNA-Binding Dye Chemistry in Droplet Digital PCR", Analytical Chemistry, vol. 85, Nov. 3, 2013, pp. 11619-11627.

Pinheiro, Leonard B. et al., "Evaluation of a Droplet Digital Polymerase Chain Reaction Format for DNA Copy Number Quantification", Analytical Chemistry, vol. 84, Nov. 28, 2011, pp. 1003-1011.

Pohl, Gudrun et al., "Principle and applications of digital PCR", Expert Review of Molecular Diagnostics, vol. 4, No. 1, 2004, pp. 41-47.

Qin, Jian et al., "Studying copy number variations using a nanofluidic platform", Nucleic Acids Research, vol. 36, No. 18, Aug. 18, 2008, pp. 1-8.

Schaerli, Yolanda et al., "The potential of microfluidic water-in-oil droplets in experimental biology", Molecular BioSystems, vol. 5, Oct. 12, 2009, pp. 1392-1404.

Therianos, Stavros et al., "Single-Channel Quantitative Multiplex Reverse Transcriptase-Polymerase Chain Reaction for Large Numbers for Gene Products Differentiates Nondemented from Neuropathological Alzheimer's Disease", American Journal of Pathology, vol. 164, No. 3, Mar. 2004, pp. 795-806.

Todorov, Tihomir et al., "A Unified Rapid PCR Method for Detection of Normal and Expanded Trinucleotide Alleles of CAG Repeats in Huntington Chorea and CGG Repeats in Fragile X Syndrome", Molecular Biotechnology, vol. 45, Mar. 9, 2010, pp. 150-154.

Wang, Weijie et al., "DNA quantification using EvaGreen and a real-time PCR instrument", Analytical Biochemistry, vol. 356, Jun. 9, 2006, pp. 303-305.

Wu, Yajun et al., "Detection of olive oil using the Evagreen real-time PCR method", European Food Research and Technology, vol. 227, Feb. 13, 2008, pp. 1117-1124.

Ye, Shu et al., "An efficient procedure for genotyping single nucleotide polymorphism", Nucleic Acids Research, vol. 29, No. 17, 2001, pp. 1-8.

Zhong, Qun et al., "Multiplex digital PCR: breaking the one target per color barrier of quantitative PCR", Lab on a Chip, vol. 11, 2011, pp. 2167-2174.

Zimmermann, Bernhard G. et al., "Digital PCR: a powerful new tool for noninvasive prenatal diagnosis?", Prenatal Diagnosis, vol. 28, Nov. 10, 2008, pp. 1087-1093.

* cited by examiner

Fig. 1

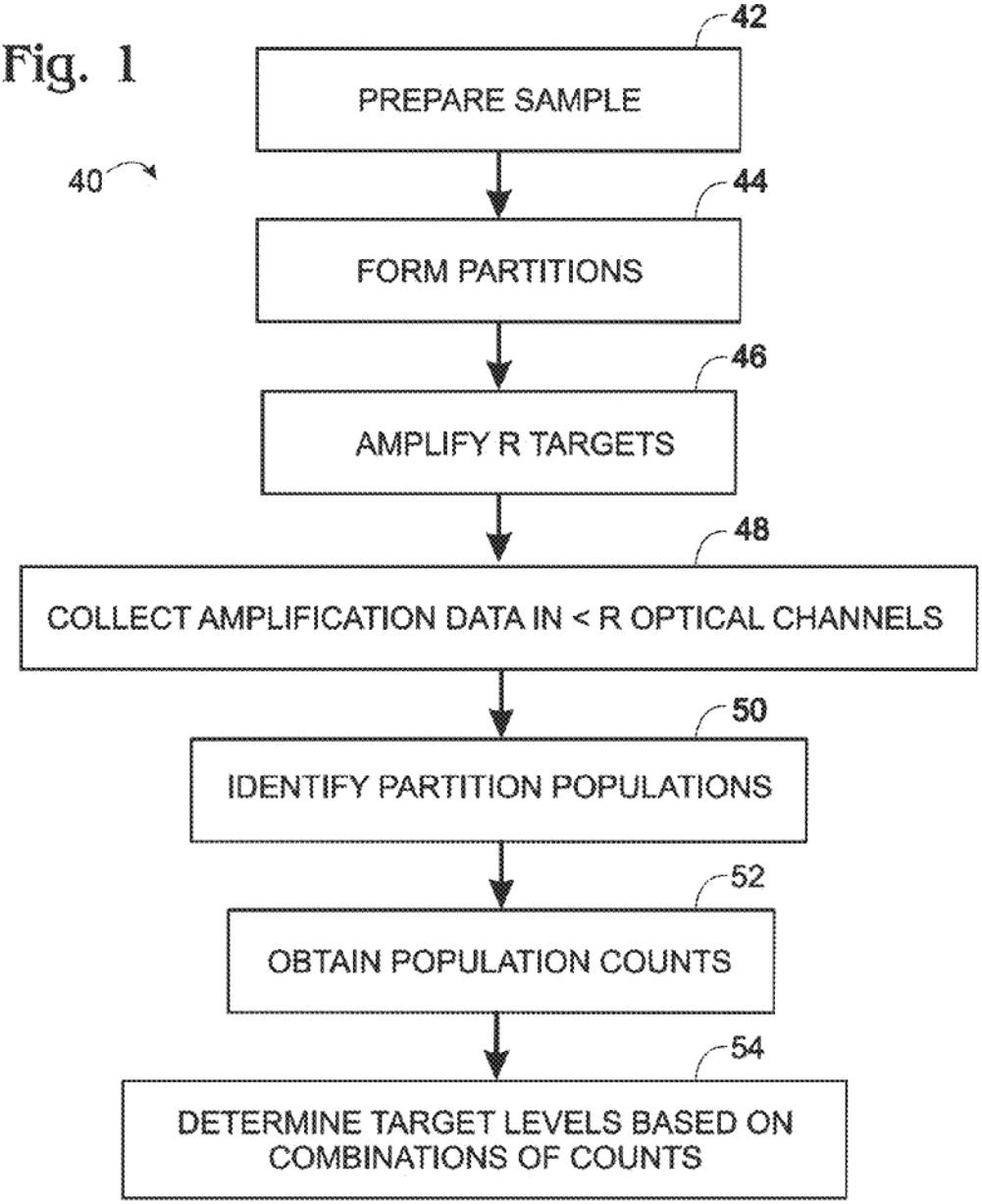

40
42
PREPARE SAMPLE
44
FORM PARTITIONS
46
AMPLIFY R TARGETS
48
COLLECT AMPLIFICATION DATA IN < R OPTICAL CHANNELS
50
IDENTIFY PARTITION POPULATIONS
52
OBTAIN POPULATION COUNTS
54
DETERMINE TARGET LEVELS BASED ON COMBINATIONS OF COUNTS

Fig. 2

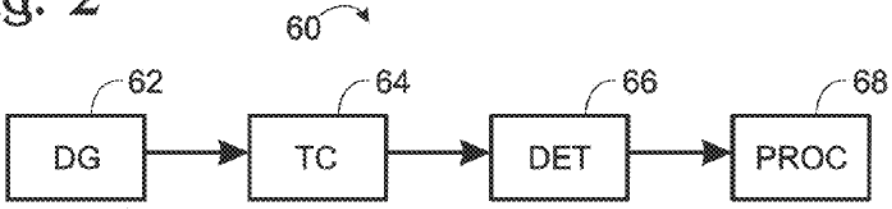

60
62
DG
64
TC
66
DET
68
PROC

Fig. 3

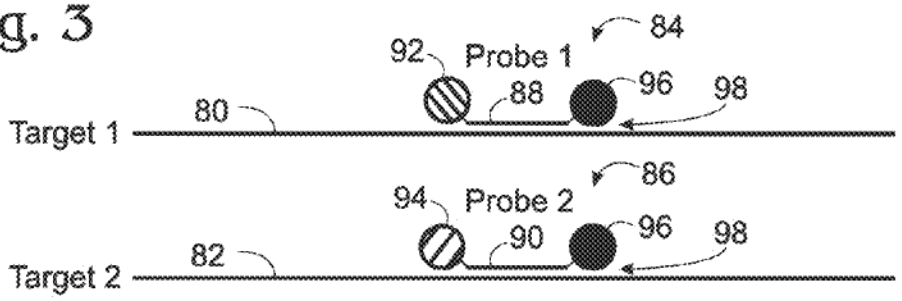



Fig. 4

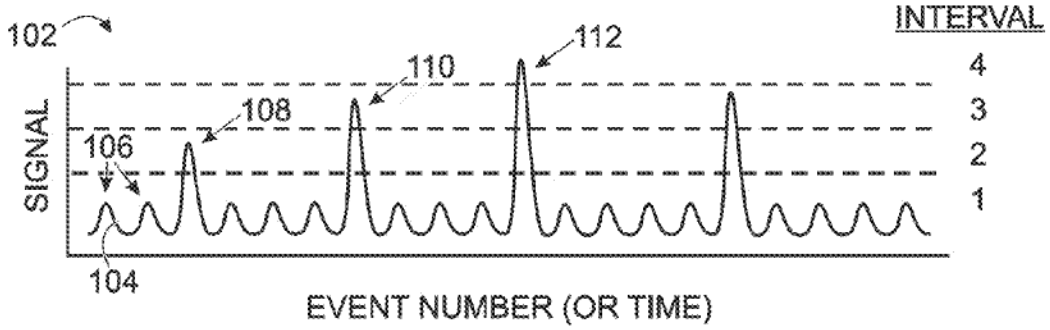



Fig. 5

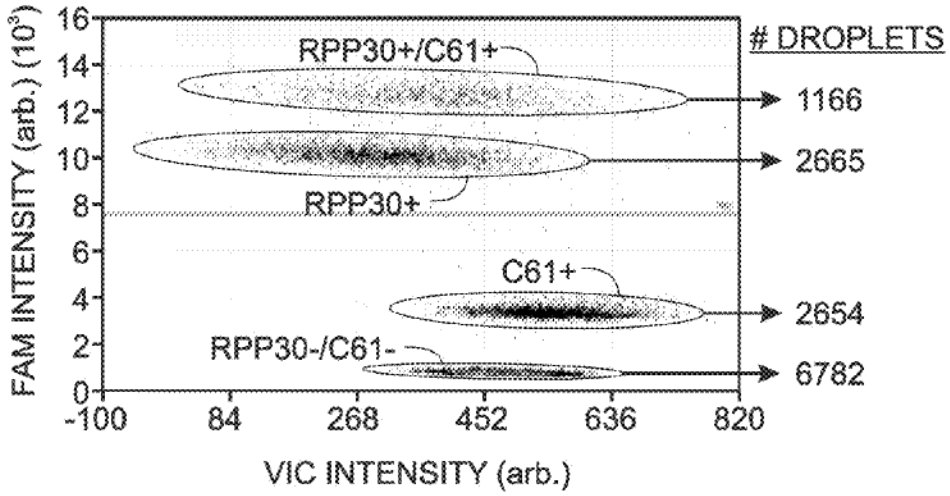


Fig. 6

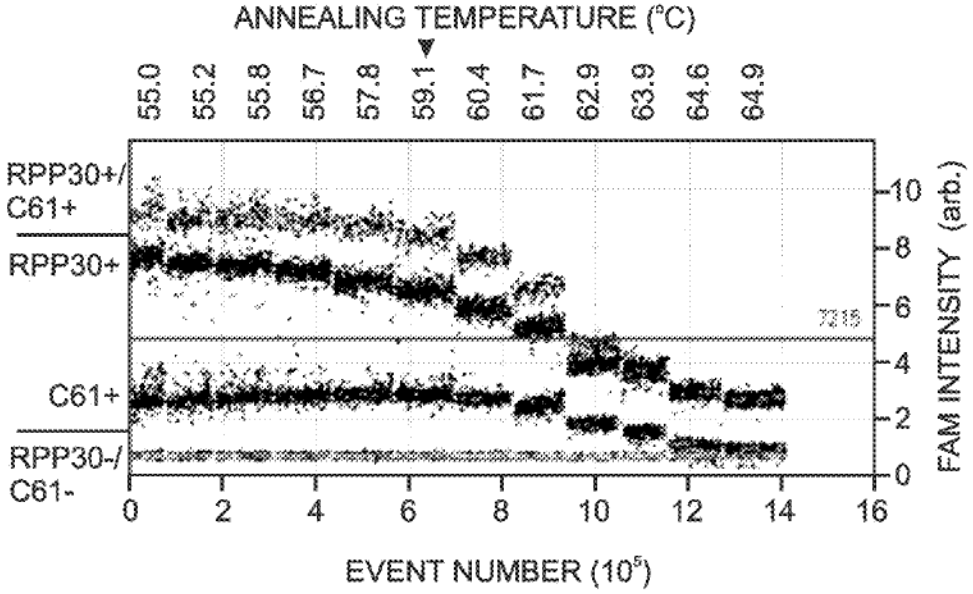

ANNEALING TEMPERATURE (°C)
55.0
55.2
55.8
56.7
57.8
59.1
60.4
61.7
62.9
63.9
64.6
64.9
RPP30+/
C61+
RPP30+
C61+
RPP30-/
C61-
7215
FAM INTENSITY (arb.)
EVENT NUMBER (10^5)

Fig. 7

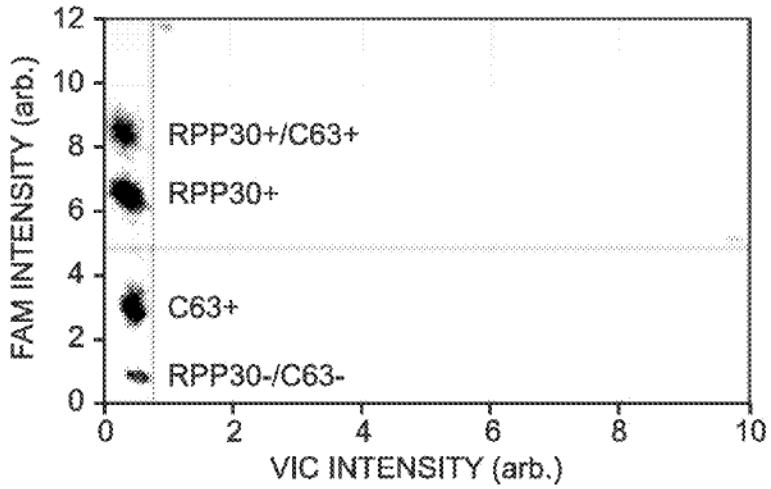

RPP30+/C63+
RPP30+
C63+
RPP30-/C63-
FAM INTENSITY (arb.)
VIC INTENSITY (arb.)

Fig. 8A

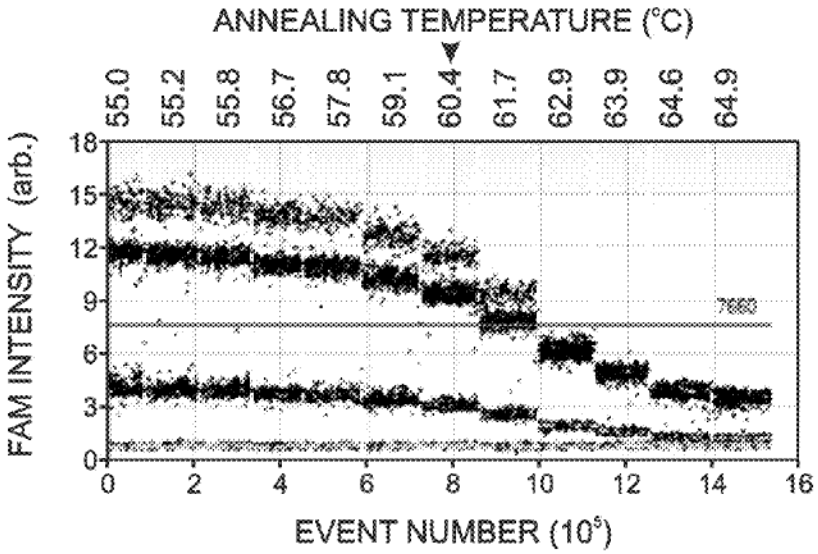

ANNEALING TEMPERATURE (°C)
55.0
55.2
55.8
56.7
57.8
59.1
60.4
61.7
62.9
63.9
64.6
64.9
FAM INTENSITY (arb.)
EVENT NUMBER (10^5)

Fig. 8B

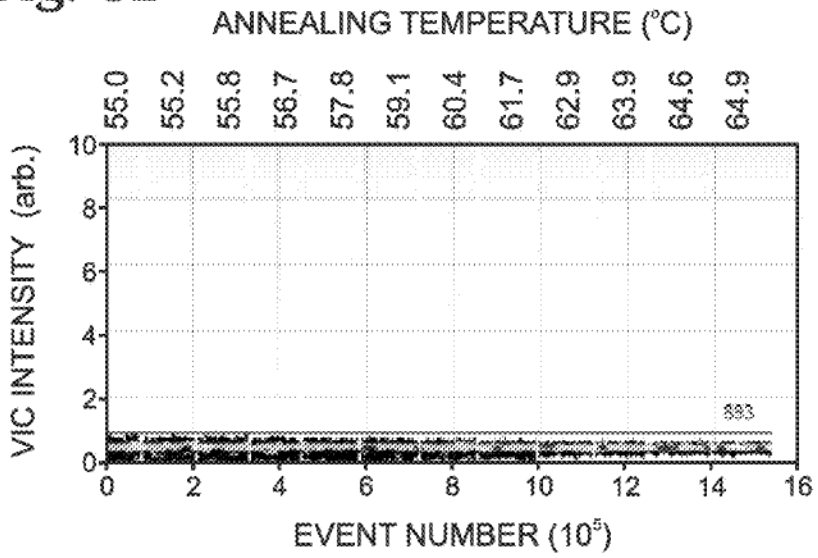

ANNEALING TEMPERATURE (°C)
55.0
55.2
55.8
56.7
57.8
59.1
60.4
61.7
62.9
63.9
64.6
64.9
VIC INTENSITY (arb.)
EVENT NUMBER (10^5)

Fig. 9

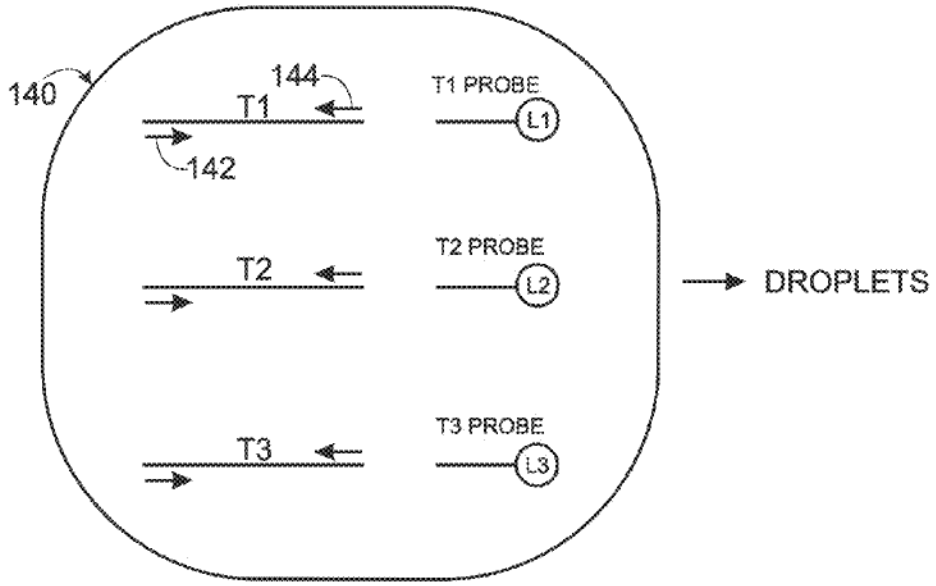

140
144
T1
142
T1 PROBE
L1
T2
T2 PROBE
L2
DROPLETS
T3
T3 PROBE
L3

Fig. 10

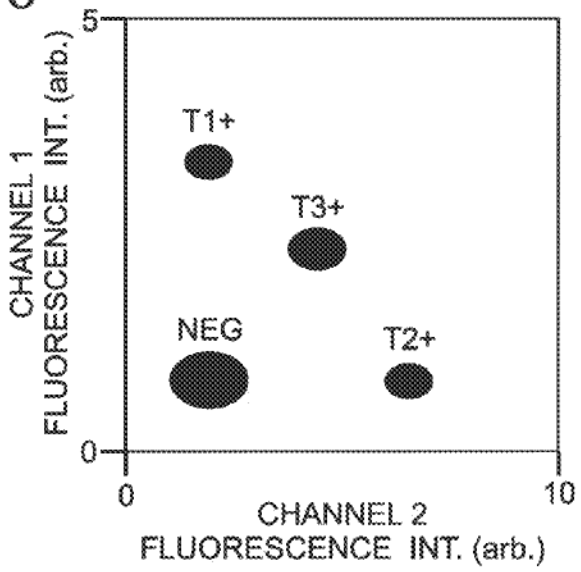

5
0
CHANNEL 1
FLUORESCENCE INT. (arb.)
T1+
T3+
NEG
T2+
0
10
CHANNEL 2
FLUORESCENCE INT. (arb.)

Fig. 11

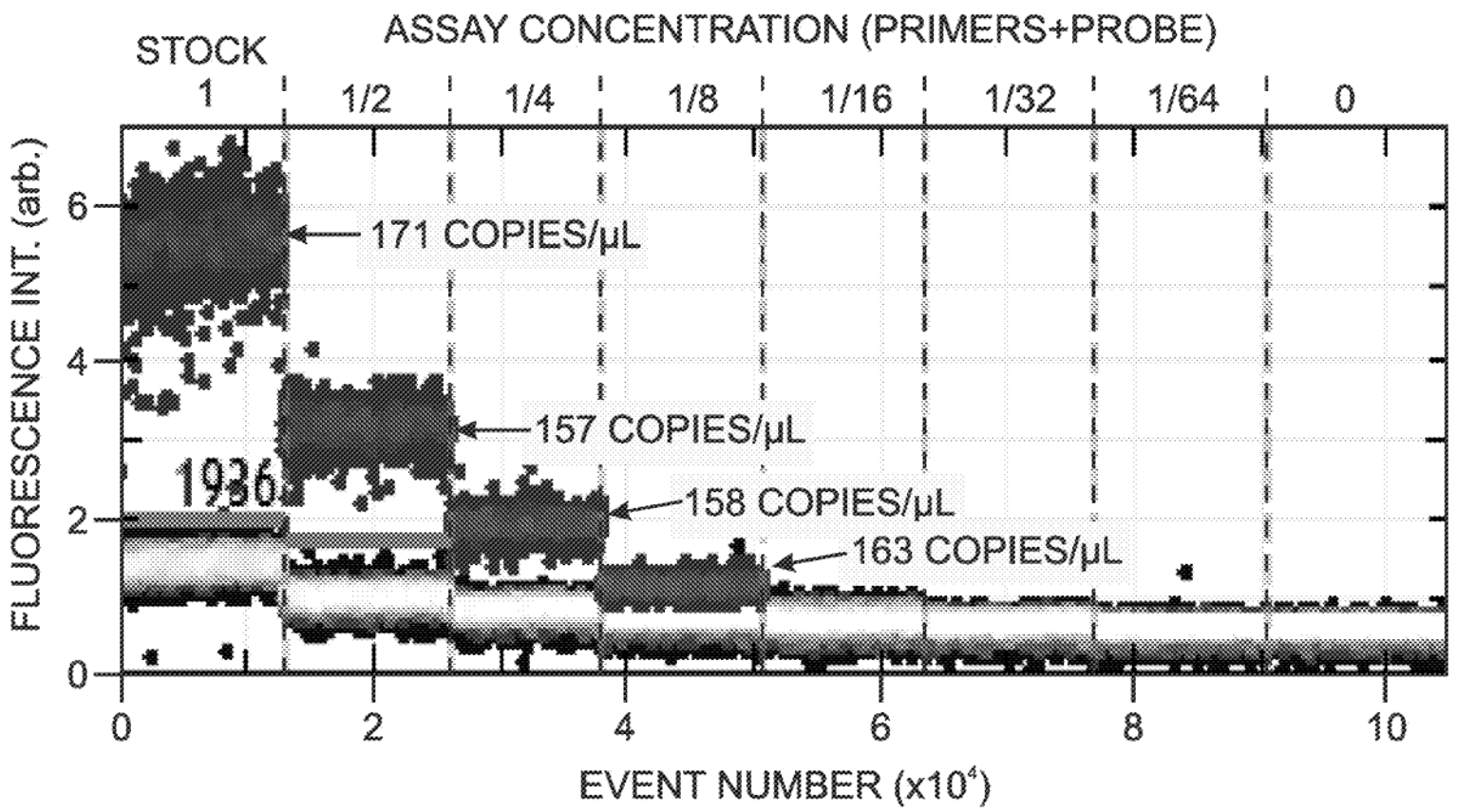

ASSAY CONCENTRATION (PRIMERS+PROBE)
STOCK
1
1/2
1/4
1/8
1/16
1/32
1/64
0
FLUORESCENCE INT. (arb.)
6
4
2
0
171 COPIES/µL
157 COPIES/µL
158 COPIES/µL
163 COPIES/µL
1936
0
2
4
6
8
10
EVENT NUMBER (x10⁴)

Fig. 12

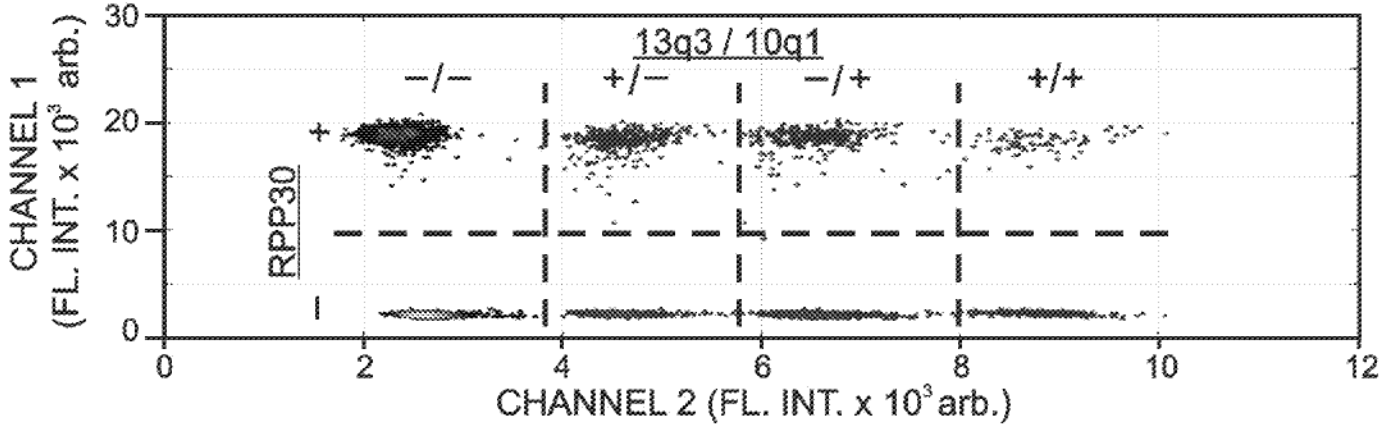

13q3 / 10q1
−/−
+/−
−/+
+/+
RPP30
+
−
CHANNEL 1 (FL. INT. x 10³ arb.)
CHANNEL 2 (FL. INT. x 10³ arb.)
0
2
4
6
8
10
12
0
10
20
30

Fig. 13

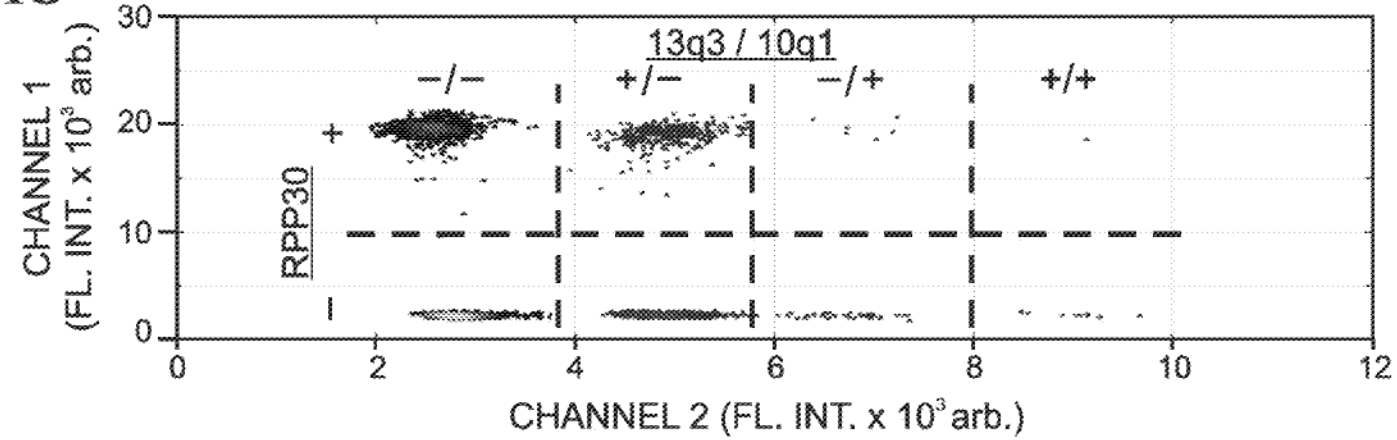

13q3 / 10q1
−/−
+/−
−/+
+/+
RPP30
+
−
CHANNEL 1 (FL. INT. x 10³ arb.)
CHANNEL 2 (FL. INT. x 10³ arb.)
0
2
4
6
8
10
12
0
10
20
30

Fig. 14

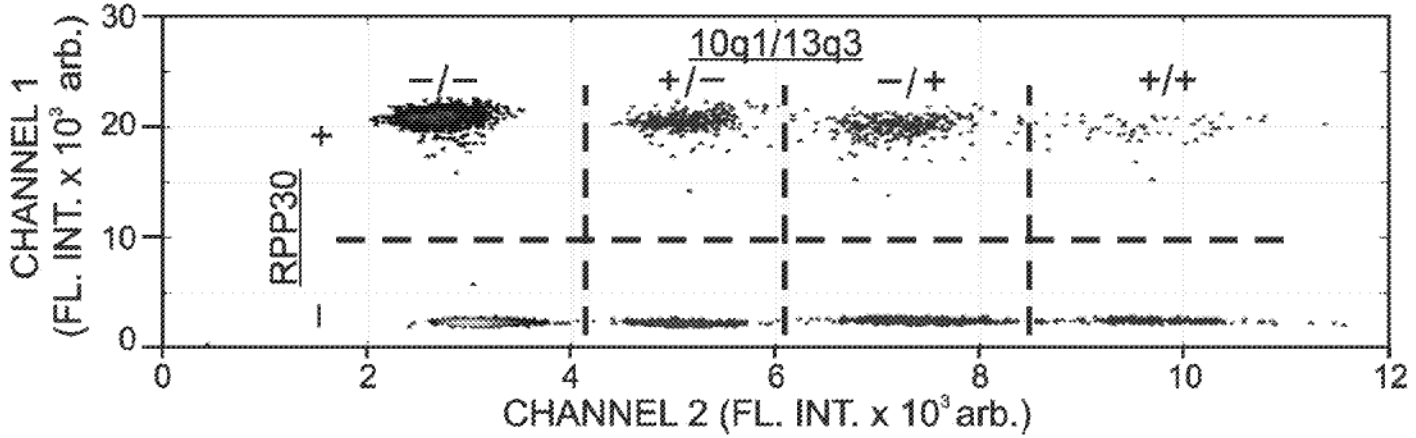

10q1/13q3
−/−
+/−
−/+
+/+
RPP30
+
−
CHANNEL 1 (FL. INT. x 10³ arb.)
CHANNEL 2 (FL. INT. x 10³ arb.)
0
10
20
30
0
2
4
6
8
10
12

Fig. 15

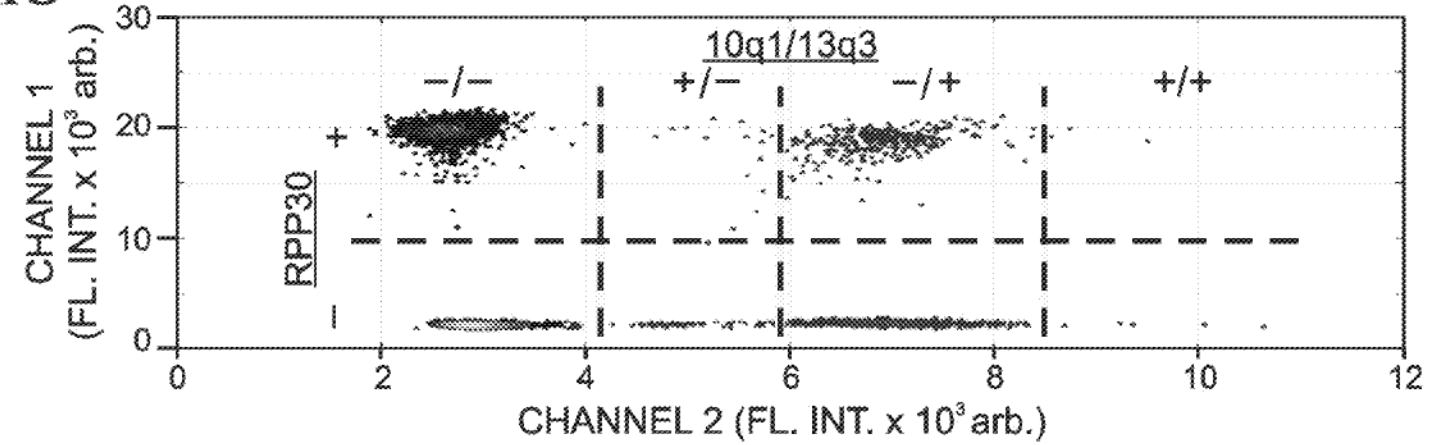
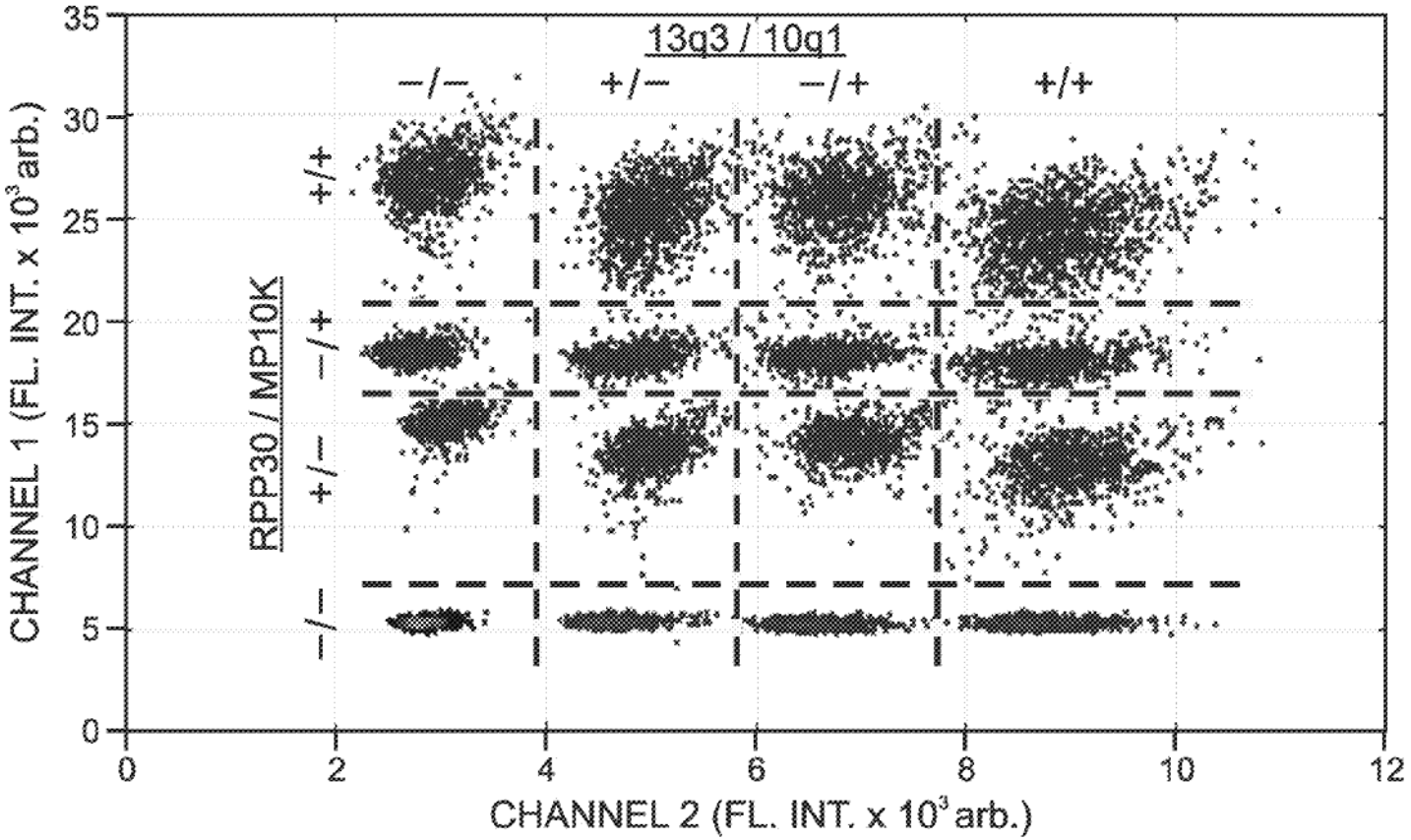

10q1/13q3
−/−
+/−
−/+
+/+
RPP30
+
−
CHANNEL 1 (FL. INT. x 10³ arb.)
CHANNEL 2 (FL. INT. x 10³ arb.)
0
10
20
30
0
2
4
6
8
10
12

Fig. 16


13q3 / 10q1
−/−
+/−
−/+
+/+
RPP30 / MP10K
+/+
−/+
+/−
−/−
CHANNEL 1 (FL. INT. x 10³ arb.)
CHANNEL 2 (FL. INT. x 10³ arb.)
0
5
10
15
20
25
30
35
0
2
4
6
8
10
12

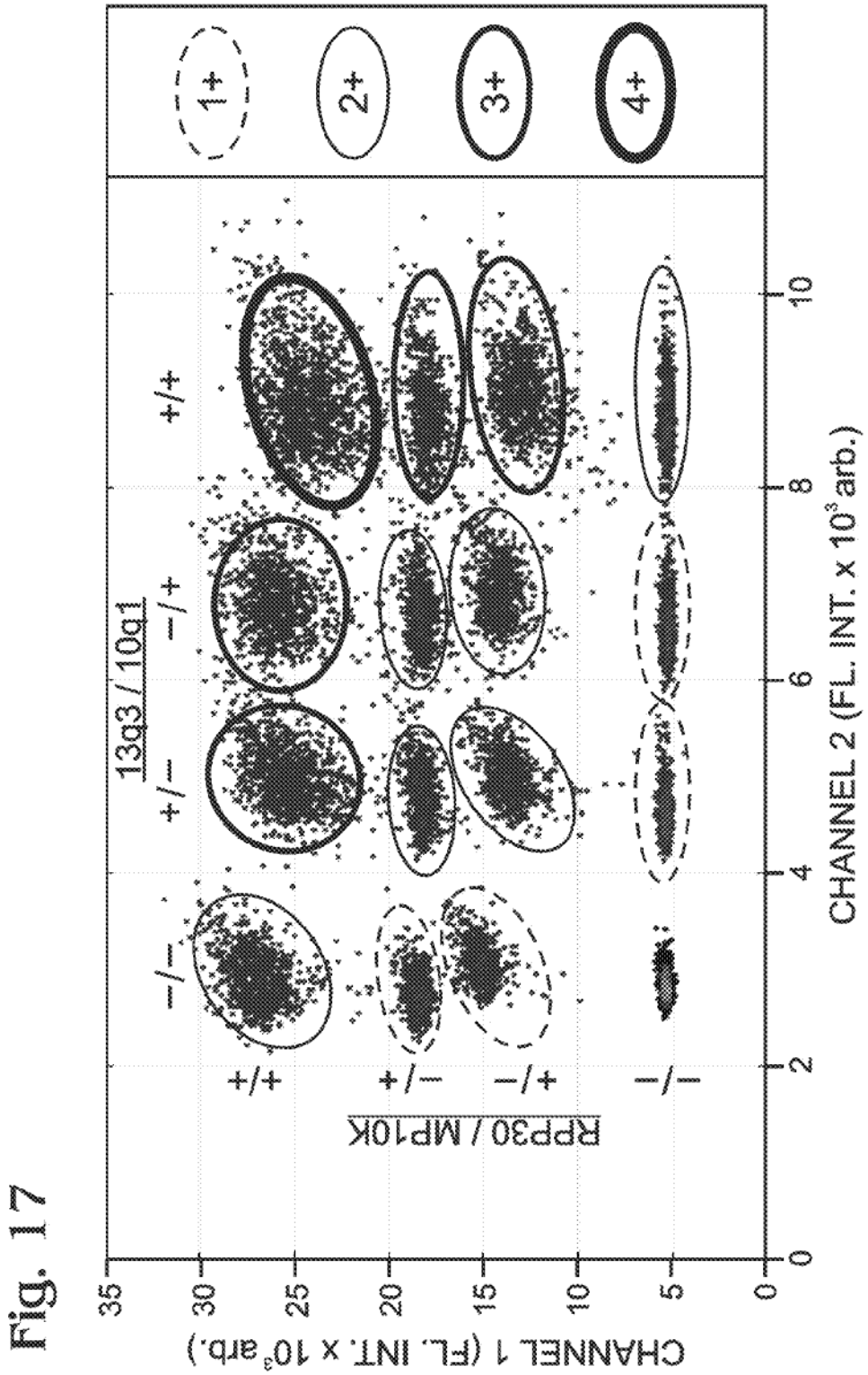

1+
2+
3+
4+
13q3 / 10q1
+/+
−/+
+/−
−/−
RPP30 / MP10K
+/+
+/−
−/+
−/−
CHANNEL 1 (FL. INT. x 10³arb.)
CHANNEL 2 (FL. INT. x 10³arb.)
0
5
10
15
20
25
30
35
0
2
4
6
8
10

Fig. 17

Fig. 18

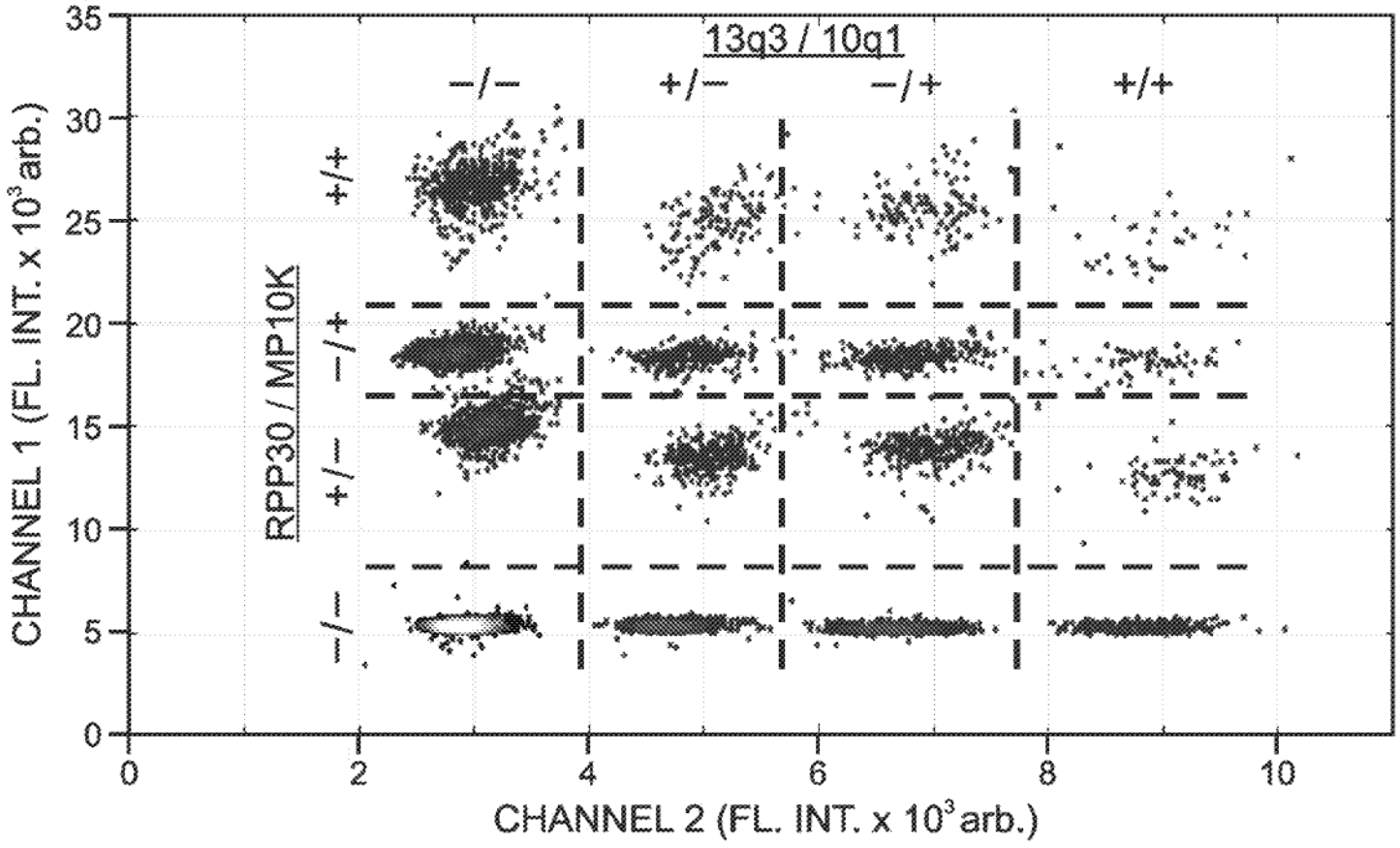
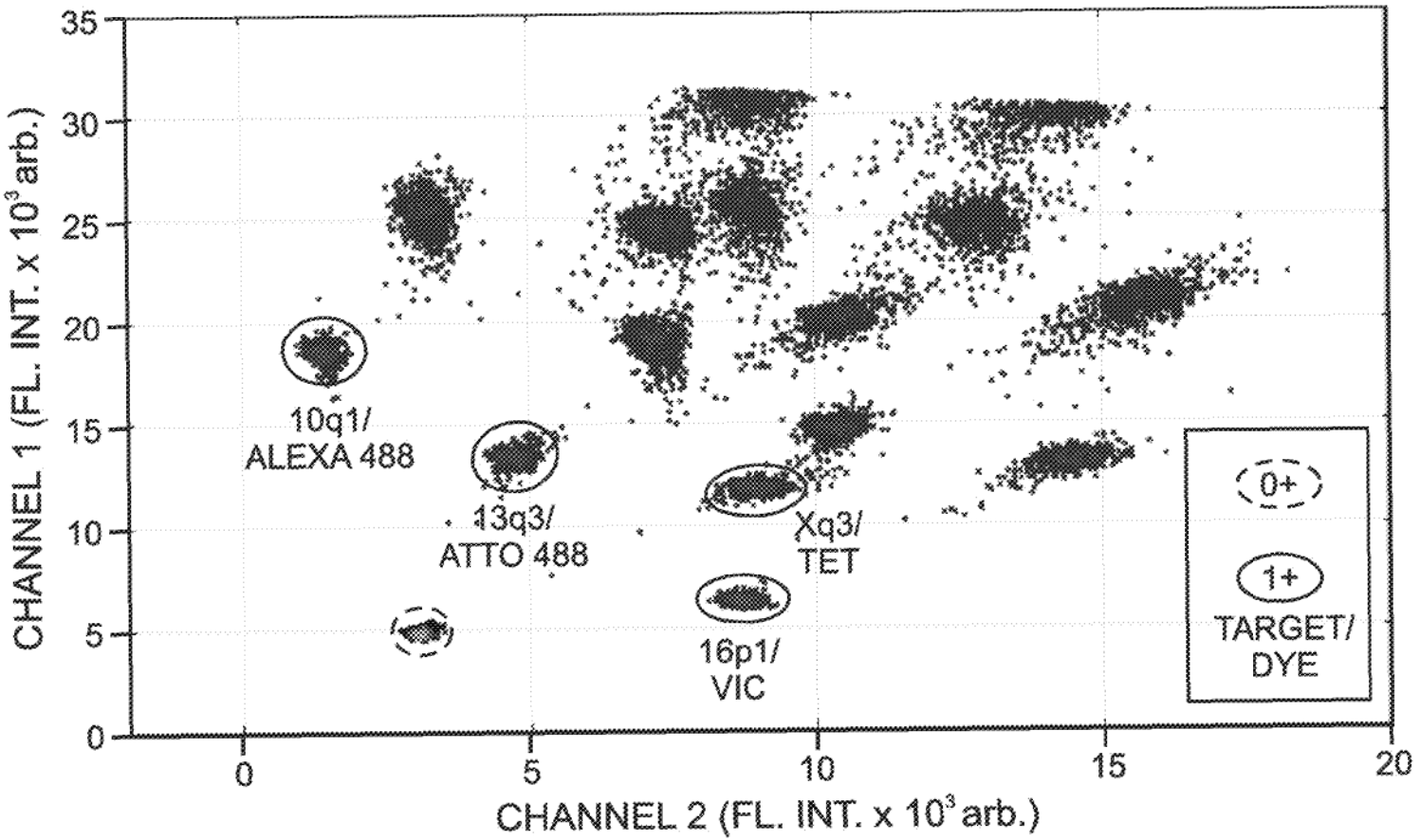

13q3 / 10q1
−/−
+/−
−/+
+/+
RPP30 / MP10K
+/+
−/+
+/−
−/−
CHANNEL 1 (FL. INT. x 10³ arb.)
CHANNEL 2 (FL. INT. x 10³ arb.)
0
5
10
15
20
25
30
35
0
2
4
6
8
10

Fig. 19


CHANNEL 1 (FL. INT. x 10³ arb.)
CHANNEL 2 (FL. INT. x 10³ arb.)
0
5
10
15
20
25
30
35
10q1/
ALEXA 488
13q3/
ATTO 488
Xq3/
TET
16p1/
VIC
0+
1+
TARGET/
DYE

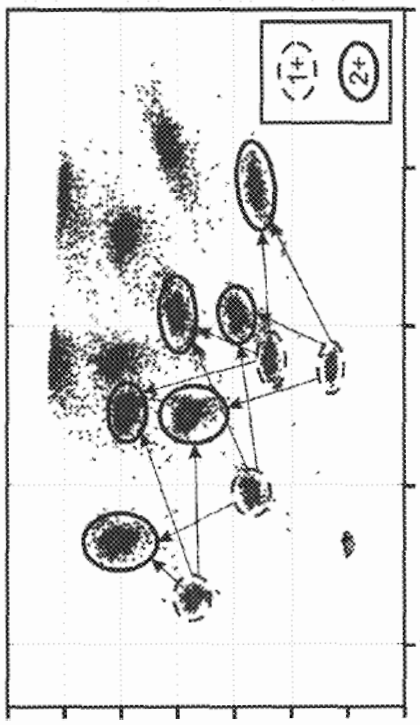



Fig. 21

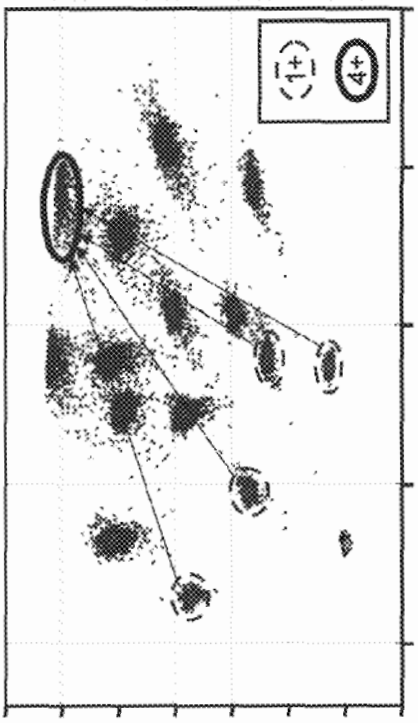



Fig. 23

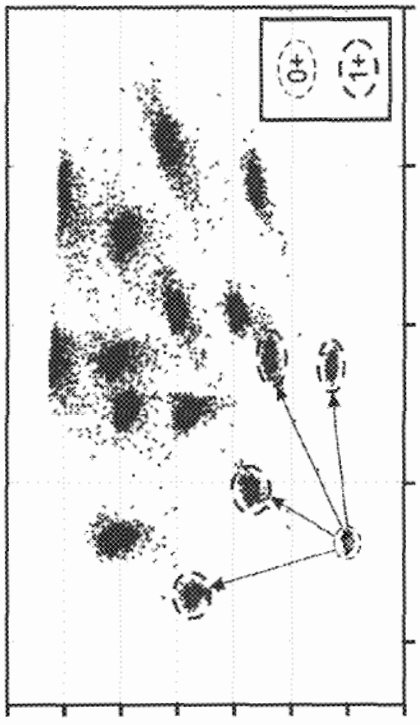



Fig. 20

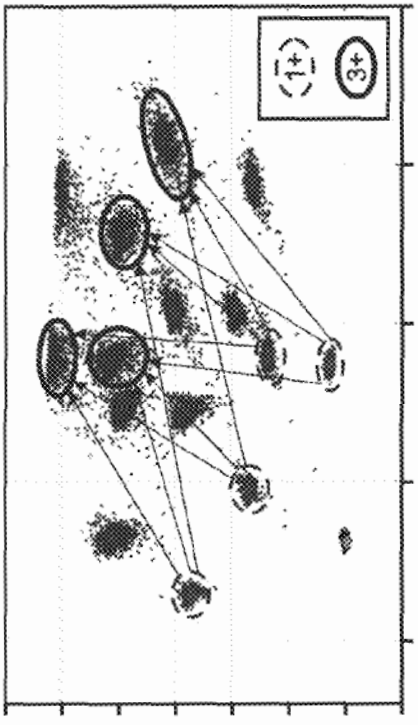
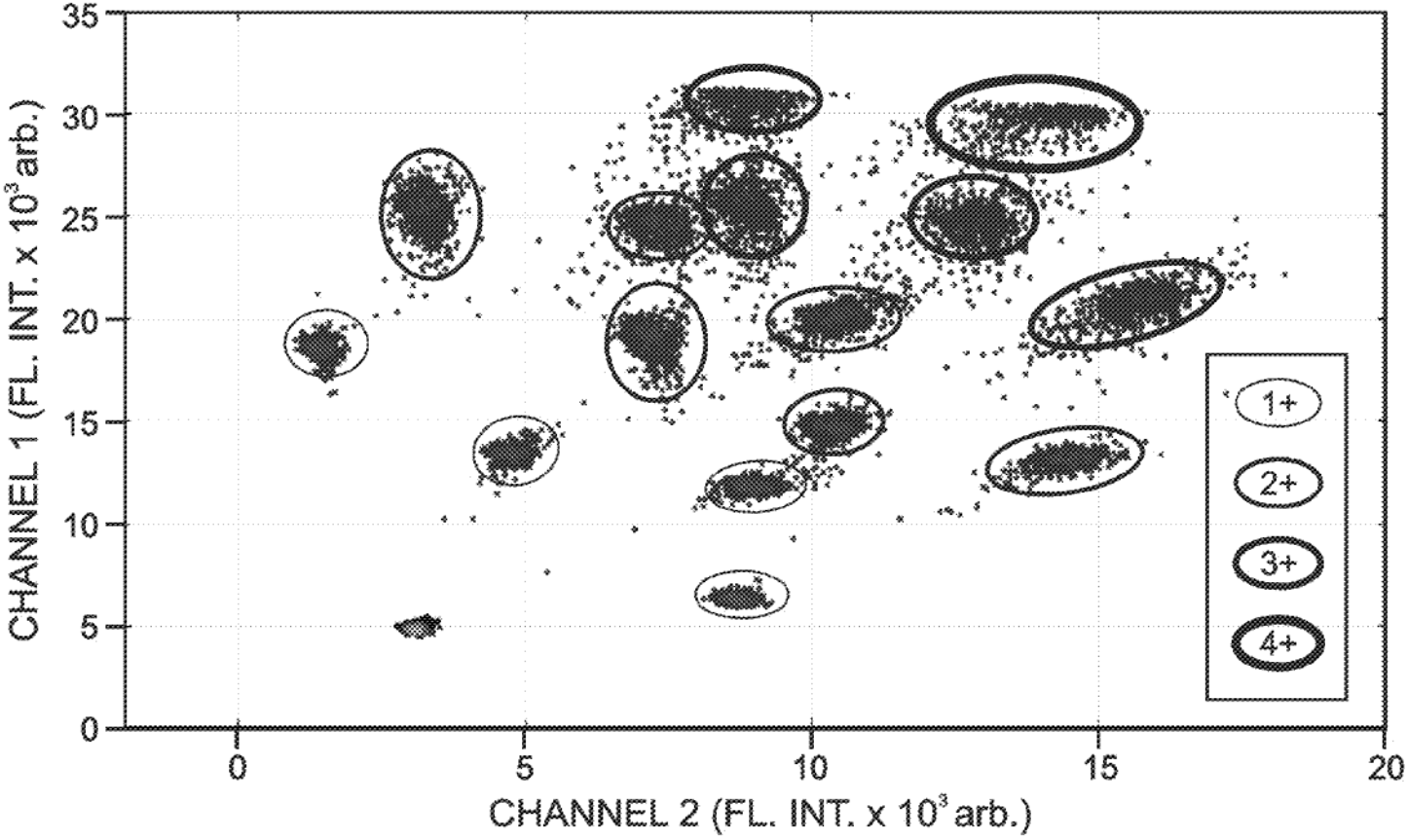



Fig. 22

Fig. 24


CHANNEL 1 (FL. INT. x 10³ arb.)
CHANNEL 2 (FL. INT. x 10³ arb.)
0
5
10
15
20
25
30
35
1+
2+
3+
4+

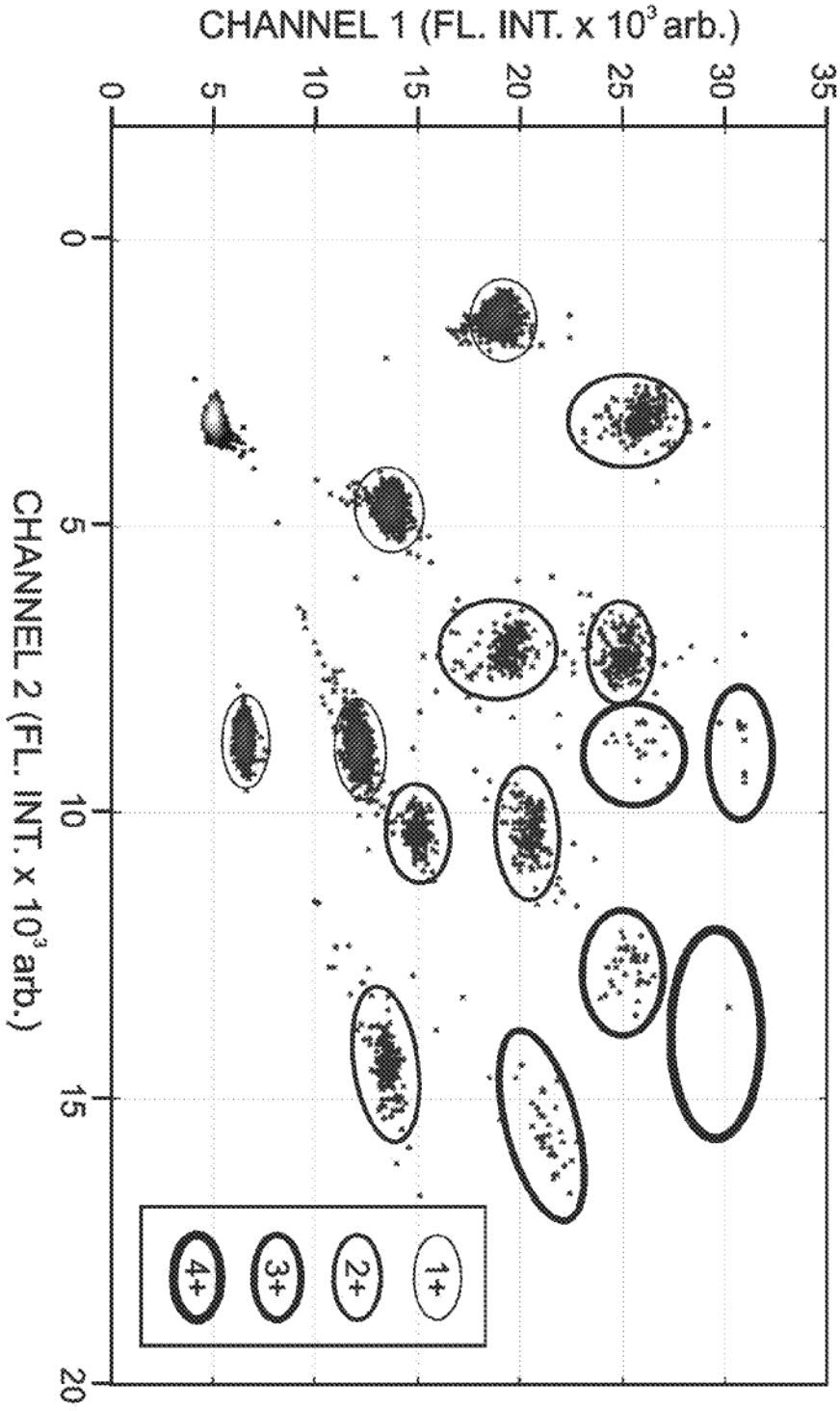

CHANNEL 1 (FL. INT. x 10³ arb.)
0
5
10
15
20
25
30
35
CHANNEL 2 (FL. INT. x 10³ arb.)
0
5
10
15
20
4+
3+
2+
1+

Fig. 25

# MULTIPLEXED DIGITAL ASSAYS

## CROSS-REFERENCES TO PRIORITY APPLICATIONS

This application is a continuation-in-part of U.S. patent application Ser. No. 13/424,304, filed Mar. 19, 2012, and U.S. patent application Ser. No. 13/548,062, filed Jul. 12, 2012, and claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Patent Application Ser. No. 61/734,296, filed Dec. 6, 2012.

U.S. patent application Ser. No. 13/424,304, in turn, is based upon and claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Patent Application Ser. No. 61/454,373, filed Mar. 18, 2011.

U.S. patent application Ser. No. 13/548,062, in turn, is based upon and claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Patent Application Ser. No. 61/507,082, filed Jul. 12, 2011; and U.S. Provisional Patent Application Ser. No. 61/510,013, filed Jul. 20, 2011.

Each of these priority applications is incorporated herein by reference in its entirety for all purposes.

## CROSS-REFERENCES TO OTHER MATERIALS

This application incorporates by reference in their entireties for all purposes the following materials: U.S. Pat. No. 7,041,481, issued May 9, 2006; U.S. Patent Application Publication No. 2010/0173394 A1, published Jul. 8, 2010; U.S. Patent Application Publication No. 2012/0194805 A1, published Aug. 2, 2012; U.S. Patent Application Publication No. 2013/0084572 A1, published Apr. 4, 2013; and Joseph R. Lakowicz, PRINCIPLES OF FLUORESCENCE SPECTROSCOPY ($2^{nd}$ Ed. 1999).

## INTRODUCTION

Digital assays generally rely on the ability to detect the presence or activity of individual copies of an analyte in a sample. In an exemplary digital assay, a sample is separated into a set of partitions, generally of equal volume, with each containing, on average, less than about one copy of the analyte. If the copies of the analyte are distributed randomly among the partitions, some partitions should contain no copies, others only one copy, and, if the number of partitions is large enough, still others should contain two copies, three copies, and even higher numbers of copies. The probability of finding exactly 0, 1, 2, 3, or more copies in a partition, based on a given average concentration of analyte in the partitions, is described by a Poisson distribution. Conversely, the concentration of analyte in the partitions (and thus in the sample) may be estimated from the probability of finding a given number of copies in a partition.

Estimates of the probability of finding no copies and of finding one or more copies may be measured in the digital assay. Each partition can be tested to determine whether the partition is a positive partition that contains at least one copy of the analyte, or is a negative partition that contains no copies of the analyte. The probability of finding no copies in a partition can be approximated by the fraction of partitions tested that are negative (the "negative fraction"), and the probability of finding at least one copy by the fraction of partitions tested that are positive (the "positive fraction"). The positive fraction or the negative fraction then may be utilized to determine the concentration of the analyte in the partitions by Poisson statistics.



Digital assays frequently rely on amplification of a nucleic acid target in partitions to enable detection of a single copy of an analyte. Amplification may be conducted via the polymerase chain reaction (PCR), to achieve a digital PCR assay. The target amplified may be the analyte itself or a surrogate for the analyte generated before or after formation of the partitions. Amplification of the target can be detected optically from a fluorescent probe included in the reaction. In particular, the probe can include a dye that provides a fluorescence signal indicating whether or not the target has been amplified.

A digital PCR assay can be multiplexed to permit detection of two or more different targets within each partition. Amplification of the targets can be distinguished by utilizing target-specific probes labeled with different dyes. However, instruments with more optical channels, to detect emission from an equivalent number of dyes, are more expensive than those with fewer channels. On the other hand, many applications, especially where sample is limited, could benefit greatly from higher degrees of multiplexing.

A new approach is needed to increase the multiplex levels of digital assays.

## SUMMARY

The present disclosure provides a system, including methods and apparatus, for performing a multiplexed digital assay.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a flowchart of an exemplary method of performing a multiplexed digital assay with more targets than optical channels for detection, in accordance with aspects of the present disclosure.

FIG. **2** is a schematic view of an exemplary apparatus for performing the multiplexed digital assay of FIG. **1**, in accordance with aspects of the present disclosure.

FIG. **3** is a schematic view of a pair of targets and corresponding probes capable of reporting the presence or absence of the targets via emitted light that may be detected together in the same channel for one or both targets in a multiplexed digital PCR assay, in accordance with aspects of the present disclosure.

FIG. **4** is an exemplary graph showing a signal that may be created by detecting light emitted from the probes of FIG. **3** in a digital PCR assay performed in droplets, with the signal created by detecting light from each target together in the same optical channel from a fluid stream containing the droplets, in accordance with aspects of the present disclosure.

FIG. **5** is an exemplary scatter plot showing droplet (or peak) intensities in arbitrary units ("arb.") for a C61/RPP30 assay, where droplet data from reporters for C61 and RPP30 are collected together in the same optical channel and plotted as a function of intensity in a FAM channel (vertical axis) (configured to detect FAM-containing probes) versus intensity in a VIC channel (horizontal axis) (configured to detect VIC-containing probes).

FIG. **6** is a graph showing FAM intensities for the C61/RPP30 assay of FIG. **5** for twelve different sets of assay conditions, namely, amplification of the C61 and RPP30 targets with different annealing temperatures (given in Celsius and denoted, from left to right, 55.0, 55.2, . . . , 64.6, and 64.9).

FIG. **7** is an exemplary scatter plot showing droplet (or peak) intensities for a C63/RPP30 droplet assay, where data

from reporters for C63 and RPP30 are collected and plotted as a function of intensity in a FAM channel (vertical axis) versus intensity in a VIC channel (horizontal axis) for each detected event (i.e., each droplet).

FIG. **8** shows FAM intensities (top panel; FIG. **8**A) and VIC intensities (bottom panel; FIG. **8**B) for the C63/RPP30 droplet assay of FIG. **7**, for twelve different sets of assay conditions, namely, amplification of the C61 and RPP30 targets with different annealing temperatures (given in degrees Celsius and denoted, from left to right, 55.0, 55.2, . . . , 64.6, and 64.9).

FIG. **9** is a schematic representation of an exemplary bulk phase mixture being separated into droplets during performance of an exemplary multiplexed digital assay for three or more distinct targets (T1, T2, and T3) using a different reporter for detecting amplification of each target (a T1 probe, a T2 probe, and a T3 probe) each including a different luminophore (L1, L2, and L3, respectively), in accordance with aspects of the present disclosure.

FIG. **10** is a schematic representation of an exemplary scatter plot showing droplet (or peak) intensities for a T1/T2/T3 droplet assay that may be performed with the droplets of FIG. **9**, where data from the T1, T2, and T3 probes are collected in a first optical channel and a second optical channel and plotted as a function of intensity in the first channel (vertical axis) versus intensity in the second channel (horizontal axis), with intensities produced by exemplary populations or clusters of negative (NEG), T1-positive (T1+), T2-positive (T2+), and T3-positive (T3+) droplets represented by filled ellipses, in accordance with aspects of the present disclosure.

FIG. **11** is a graph of fluorescence intensity data collected in a one-target amplification assay performed in droplets, at the indicated assay concentration for primers and probe, to illustrate how the assay concentration can be modified to increase or decrease the level of the intensity signal for each amplification-positive (and/or negative) droplet (or "event"), in accordance with aspects of the present disclosure.

FIG. **12** is a 2-D scatter plot of fluorescence intensity ("FL. INT.") data obtained from individual droplets detected in two optical channels during performance of a triplex amplification assay for three targets (RPP30, Chr13q3, and Chr10q1), with the sample (template DNA) digested before amplification with a restriction enzyme (AluI) that does not cut within any of the templates for the targets, and with the target composition of each droplet population indicated ("–" is negative and "+" is positive for each indicated target), in accordance with aspects of the present disclosure.

FIG. **13** is another 2-D scatter plot of fluorescence intensity data obtained generally as in FIG. **12** but with the sample (template DNA) digested, before amplification, with a restriction enzyme (HaeIII) that cuts between the priming sites of the Chr10q1 template, in accordance with aspects of the present disclosure.

FIG. **14** is a 2-D scatter plot of fluorescence intensity data obtained from individual droplets detected in two optical channels during performance of a triplex amplification assay as in FIG. **12**, but with the concentrations of the Chr13q3 and Chr10q1 assays adjusted to changes the endpoint fluorescence level for assay of these targets, such that the relative positions of the corresponding single-positive populations are inverted in the plot, in accordance with aspects of the present disclosure.

FIG. **15** is another 2-D scatter plot of fluorescence intensity data obtained with the assay concentrations of FIG. **14**, but with the sample (template DNA) digested, before amplification, with a restriction enzyme (HaeIII) that cuts between the priming sites of the Chr10q1 template, in accordance with aspects of the present disclosure.

FIG. **16** is a 2-D scatter plot of fluorescence intensity data obtained from individual droplets detected in two optical channels during performance of a tetraplex amplification assay for four targets (RPP30, MP10K, Chr13q3, and Chr10q1), with the sample (template DNA) digested before amplification with a restriction enzyme (AluI) that does not cut within any of the templates for the targets, and with the resulting droplet populations separated by dashed lines and the target composition of each droplet population indicated ("–" is negative and "+" is positive for each indicated target), in accordance with aspects of the present disclosure.

FIG. **17** is the 2-D scatter plot of FIG. **16**, with the dashed lines removed and each droplet population circled using a line weight that indicates the number of different targets for which each population is amplification positive, in accordance with aspects of the present disclosure.

FIG. **18** is a 2-D scatter plot of fluorescence intensity data obtained with the tetraplex assay of FIG. **16**, but with the sample (template DNA) diluted by a factor of four relative to FIG. **16**, in accordance with aspects of the present disclosure.

FIG. **19** is a 2-D scatter plot of fluorescence intensity data obtained from individual droplets detected in two optical channels during performance of a tetraplex amplification assay for four targets (Chr10q1, Chr13q3, Chr16 µl, and ChrXq3) using four corresponding probes each containing a different fluorophore (Alexa Fluor® 488 dye, Atto® 488 dye, VIC dye, and TET dye, respectively), with each single-positive population of droplets labeled, in accordance with aspects of the present disclosure.

FIGS. **20-23** each reproduce the scatter plot of FIG. **19** and respectively show single-, double-, triple-, and quadruple-positive droplet populations as circled, with arrows indicating the distinct target composition of each population, in accordance with aspects of the present disclosure.

FIG. **24** is a composite of FIGS. **20-23** that marks all of the target-positive droplet populations in the same plot, in accordance with aspects of the present disclosure.

FIG. **25** is a 2-D scatter plot obtained as in FIGS. **19** and **24** but with only one-sixth as much template DNA, in accordance with aspects of the present disclosure.

## DETAILED DESCRIPTION

The present disclosure provides a system, including methods and apparatus, for performing a multiplexed digital assay.

The present disclosure provides a system, including methods and apparatus, for performing a digital assay on a potentially greater number of targets through multiplexed detection of signals from reporters for two or more distinct targets in a common or shared channel ("the same optical channel"). The reporters may include the same fluorophore, such as FAM or VIC, or different fluorophores with similar spectral characteristics, so that light from two or more reporters can be collected simultaneously in the same optical channel. The assays may be constructed so that data for each target are distinguishable, for example, by choosing assays for each target that have sufficiently distinct endpoints (or time courses). The contents of each sample or sample partition may then be determined: those with no targets, those with a first assay (assay 1) target, those with a second assay (assay 2) target, and those with both, in a two-target assay. The total number of droplets positive for each target

(e.g., target 1 and target 2) can be estimated by taking into account the number of droplets in each population. A concentration for each target may be estimated based on the number of droplets positive for each target and the total number of droplets, for example, using Poisson statistics. Moreover, the relative numbers of different targets (including reference targets) may be estimated, allowing determination of copy number (CN), copy number variation (CNV), and the presence/abundance of single nucleotide polymorphisms (SNPs), among other quantities. Copy number represents the number of copies of a given target present in a genome (e.g., humans have a diploid genome with a copy number of two for most autosomal genes). Copy number variation is a structural variation in the genome, such as deletions, duplications, translocations, and/or inversions, which may be a major source of heritable genetic variation, including susceptibility to disease (or disease itself) and responsiveness to disease treatment.

The assays may be extended in various ways. In some embodiments, the assays may involve detection of more than two targets in the same optical channel. For example, an assay for three targets may generate eight clusters or populations of data, separated by intensity. In the same or other embodiments, some targets may be analyzed in one channel (e.g., a FAM channel), and one or more other targets may be analyzed in one or more other channels (e.g., a VIC channel). Three targets, two in a first channel and one in a second channel, would again generate eight clusters or populations of data, but they would be separated in a two-dimensional intensity space and so in principle more easily resolvable.

A method of performing a multiplexed digital assay is provided. In the method, droplets may be formed that collectively contain R targets. The targets may be amplified in the droplets. Data may be collected that represents amplification of the targets in the droplets. The data may be collected in fewer than R optical channels. A level of each target may be determined with the data, wherein the level determined for at least one of the targets is based in part on a droplet count for a droplet population identified as positive for at least two of the targets.

Another method of performing a multiplexed digital assay is provided. In the method, droplets may be formed that collectively contain R targets, where R is at least three. A significant number of the droplets may contain a copy of each of two or more of the targets. The targets may be amplified in the droplets. Data may be collected representing amplification of the targets in the droplets. The data may be collected in fewer than R optical channels each representing a distinct waveband of emitted light. A plurality of droplet populations may be identified from the data, wherein forming, amplifying, and collecting are performed such that each droplet population positive for two of the targets is at least substantially resolved from each droplet population positive for only one of the targets. A level of each target may be determined based in part on one or more droplet populations identified as containing a pair of the targets.

Yet another method of performing a multiplexed digital assay is provided. In the method R targets may be amplified in droplets. Data representing amplification of the targets in the droplets may be collected. The data may be collected in a plurality of less than R optical channels each representing a distinct waveband of light. A plurality of droplet populations may be identified from the data, with each population containing a different combination of the targets. A level of each target may be determined. The step of determining for each target may be based on a value for droplet counts obtained from a combination of at least two droplet populations that excludes at least two other droplet populations.

Still another method of performing a digital assay is provided. In the method, droplets may be formed that collectively contain R targets and a probe corresponding to each target. R may be at least three, and each probe may include a different luminophore. The targets may be amplified in the droplets. Data may be collected that represents amplification of the targets in the droplets. The data may be collected in less than R optical channels each representing a distinct waveband of light. A level of each target may be determined based on the data.

Further aspects of the present disclosure are presented in the following sections: (I) system overview, and (II) examples.

I. System Overview

This section provides an overview of exemplary methods and apparatus for performing digital assays, in accordance with aspects of the present disclosure.

FIG. **1** shows a flowchart of an exemplary method **40** of performing a multiplexed digital assay. The steps presented for method **40** may be performed in any suitable order and in any suitable combination. Furthermore, the steps may be combined with and/or modified by any other suitable steps, aspects, and/features of the present disclosure.

Sample preparation. A sample may be prepared for the assay, indicated at **42**. Preparation of the sample may include any suitable manipulation of the sample, such as collection, dilution, concentration, purification, lyophilization, freezing, extraction, combination with one or more assay reagents, performance of at least one preliminary reaction to prepare the sample for one or more reactions in the assay, or any combination thereof, among others. Preparation of the sample may include rendering the sample competent for subsequent performance of one or more reactions, such as one or more enzyme catalyzed reactions and/or binding reactions.

In some embodiments, preparation of the sample may include combining the sample with reagents for amplification and for reporting whether or not amplification occurred. Reagents for amplification may include any combination of primers for the targets, dNTPs and/or NTPs, at least one enzyme (e.g., a polymerase, a ligase, a reverse transcriptase, a restriction enzyme, or a combination thereof, each of which may or may not be heat-stable), and/or the like. Accordingly, preparation of the sample may render the sample (or partitions thereof) capable of amplification of each of one or more targets, if present, in the sample (or a partition thereof). Reagents for reporting may include a different reporter for each target of interest. Accordingly, preparation of the sample for reporting may render the sample capable of reporting, or being analyzed for, whether or not amplification has occurred, on a target-by-target basis, and optionally the extent of any such amplification. The reporters each may be a labeled probe that includes a nucleic acid (e.g., an oligonucleotide) labeled with a luminophore (i.e., a photoluminescent moiety), such as a fluorophore.

Sample partitioning. The sample may be separated into partitions, indicated at **44**. Separation of the sample may involve distributing any suitable portion including up to all of the sample to the partitions. Each partition may be and/or include a fluid volume that is isolated from the fluid volumes of other partitions. The partitions may be isolated from one another by a fluid phase, such as a continuous phase of an emulsion, by a solid phase, such as at least one wall of a container, or a combination thereof, among others. In some embodiments, the partitions may be droplets disposed in a

continuous phase, such that the droplets and the continuous phase collectively form an emulsion.

The partitions may be formed by any suitable procedure, in any suitable manner, and with any suitable properties. For example, the partitions may be formed with a fluid dispenser, such as a pipette, with a droplet generator, by agitation of the sample (e.g., shaking, stirring, sonication, etc.), and/or the like. Accordingly, the partitions may be formed serially, in parallel, or in batch. The partitions may have any suitable volume or volumes. The partitions may be of substantially uniform volume or may have different volumes. Exemplary partitions having substantially the same volume are monodisperse droplets. Exemplary volumes for the partitions include an average volume of less than about 100, 10 or 1 μL, less than about 100, 10, or 1 nL, or less than about 100, 10, or 1 pL, among others.

The partitions, when formed, may be competent for performance of one or more reactions in the partitions. Alternatively, one or more reagents may be added to the partitions after they are formed to render them competent for reaction. The reagents may be added by any suitable mechanism, such as a fluid dispenser, fusion of droplets, or the like.

The partitions collectively, before amplification, may contain copies of a plurality of targets. Each partition may contain, on average, less than about five, four, three, or two copies, or less than about one copy of at least one target and/or of each target per partition. In any event, a significant number of the partitions (e.g., at least about 1%, 2%, 5%, 10%, or 20%, among others, of the partitions) may contain a copy of each of at least two targets, and/or a plurality of the partitions each may contain a copy of all targets.

The partitions when provided (e.g., when formed) may contain each target at "partial occupancy," which means that a subset (one or more) of the partitions contain no copies of the target and the rest of partitions contain at least one copy of the target. For example, another subset (one or more) of the partitions may contain a single copy (only one copy) of the target, and, optionally, yet another subset (one or more) of the partitions (e.g., the rest of the partitions) may contain two or more copies of the target. The term "partial occupancy" permits but does not require a dilution of the sample/reaction mixture providing the target, and is not restricted to the case where there is no more than one copy of the target in any partition. Accordingly, partitions containing the target at partial occupancy may, for example, contain an average of more than, or less than, about one copy, two copies, or three copies, among others, of the template/target per partition when the partitions are provided or formed. Copies of the target may have a random distribution among the partitions, which may be described as a Poisson distribution.

Target amplification. A plurality ("R") of targets may be amplified in the partitions. (R may be 2, 3, 4, or more than 4.) Amplification of each target may occur selectively (and/or substantially) in only a subset of the partitions, such as less than about one-half, one-fourth, or one-tenth of the partitions, among others. Amplification of each target may occur selectively in partitions containing at least one copy of the target (i.e., containing at least one copy of a template corresponding to the target).

Amplification may or may not be performed isothermally. In some cases, amplification in the partitions may be encouraged by heating the partitions and/or incubating the partitions at a temperature above room temperature, such as at a denaturation temperature, an annealing temperature, and/or an extension temperature, for one or a plurality of cycles. In some examples, the partitions may be thermally cycled to promote a polymerase chain reaction and/or ligase chain reaction. Exemplary isothermal amplification approaches that may be suitable include nucleic acid sequence-based amplification, transcription-mediated amplification, multiple-displacement amplification, strand-displacement amplification, rolling-circle amplification, loop-mediated amplification of DNA, helicase-dependent amplification, and single-primer amplification, among others.

Data collection. Amplification data may be collected, indicated at **48**. The data may be collected in less than "R" optical channels. In other words, the number (R) of targets assayed may be greater than the number of optical channels used for detecting target amplification. In some cases, the data may be collected in only one or two optical channels, or in at least two, three, or more optical channels, among others.

An optical channel may represent a particular detection regime with which emitted light is generated and detected. The detection regime may be characterized by a waveband (i.e., a wavelength regime) for detection of emitted light. If pulsed excitation light is used in the detection regime to induce light emission, the detection regime may be characterized by a wavelength or waveband for illumination with excitation light and/or a time interval during which light emission is detected with respect to each light pulse. Accordingly, optical channels that are different from each other may differ with respect to the wavelength/waveband of excitation light, with respect to the wavelength/waveband of emitted light that is detected, and/or with respect to the time interval during which emitted light is detected relative to each pulse of excitation light, among others.

Data collection may include generating one or more signals representative of detected light. The signal may represent an aspect of light, such as the intensity of the light, detected in the same optical channel from reporters for two or more distinct targets. The signals optionally may include data collected in two or more different optical channels (e.g., in different wavelength ranges (wavebands) and/or color regimes) from reporters for the same and/or different targets). The light detected from each reporter may be light emitted from a luminophore (e.g., a fluorophore). The light detected in a given channel may be detected such that light from different reporters is summed or accumulated without attribution to a particular reporter. Thus, the signal for a given channel may be a composite signal that represents two, three, four, or more assays and thus two, three, four, or more targets.

The signal(s) may be created based on detected light emitted from one or more reporters in the partitions. The one or more reporters may report whether at least one of two or more particular amplification reactions represented by the signal has occurred in a partition and thus whether at least one copy of at least one of two or more particular targets corresponding to the two or more particular amplification reactions is present in the partition. The level or amplitude of the signal corresponding to the reporters may be analyzed to determine whether or not at least one of the particular amplification reactions has occurred and at least one copy of one of the particular targets is present. The level or amplitude of the signal may vary among the partitions according to whether at least one of the particular amplification reactions occurred or did not occur and at least one of the particular targets is present or absent in each partition. For example, a partition positive for a particular target may produce a signal level or amplitude that is above a given threshold and/or within a given range. Partitions may be analyzed and signals created at any suitable time(s). Exem-

plary times include at the end of an assay (endpoint assay), when reactions have run to completion and the data no longer are changing, or at some earlier time, as long as the data are sufficiently and reliably separated.

The reporters may have any suitable structure and characteristics. Each reporter may be a probe including an oligonucleotide and a luminophore associated with the oligonucleotide (e.g., with the luminophore covalently attached to the oligonucleotide), to label the oligonucleotide. The probe also may or may not include an energy transfer partner for the luminophore, such as a quencher or another luminophore. The probe may be capable of binding specifically to an amplicon produced by amplification of a target for the probe. The probe may or may not also function as a primer in the assay. Exemplary labeled probes include TaqMan® probes, Scorpion® probes/primers, Eclipse® probes, Amplifluor® probes, molecular beacon probes, Lux® primers, proximity-dependent pairs of hybridization probes that exhibit FRET when bound adjacent one another on an amplicon, QZyme® primers, or the like.

In some cases, at least one of the reporters may be a dye that interacts with (e.g., binds) nucleic acid relatively nonspecifically. For example, the dye may not be attached to an oligonucleotide that confers substantial sequence binding specificity. The dye may be a major groove binder, a minor groove binder, an intercalator, or an external binder, among others. The dye may interact preferentially with double-stranded relative to single-stranded nucleic acid and/or may exhibit a greater change in a photoluminescent characteristic (e.g., intensity) when interacting with double-stranded relative to single-stranded nucleic acid. Exemplary dyes that may be suitable include luminescent cyanines, phenanthridines, acridines, indoles, imidazoles, and the like, such as DAPI, Hoechst® 33258 dye, acridine orange, etc. Exemplary intercalating dyes that may be suitable include ethidium bromide, propidium iodide, EvaGreen® dye, SYBR® Green dye, SYBR® Gold dye, and 7-aminoactinomycin D (7-AAD), among others.

Population identification. Partition populations each positive for a different combination of zero, one, or more of the R targets may be identified, indicated at **50**. In some cases every combination of positives may be identified, and in other cases only a subset of all possible combinations may be identified (e.g., identifying single and double-positive populations, while ignoring rarer triple-positive and/or higher order populations.) Identification may be performed by a data processor using an algorithm (e.g., an algorithm that identifies patterns (e.g., droplets clusters) in the data), by a user, or a combination thereof. In some cases, a data processor may produce and output (e.g., display) a plot of the collected data (e.g., a 2-D scatter plot or histogram (e.g., see Examples 6 and 7), or, with three or more optical channels for detection, a series of 2-D scatter plots or histograms with different pairs of axes). The user then may define the boundary of each population based on the plot(s), e.g., through a graphical user interface to define population boundaries, and/or by inputting values (e.g., representing intensity ranges) to define a boundary for each population. Each population boundary may be defined by one or more ranges of values, a geometrical shape that surrounds the population (e.g., a polygon, ellipse, etc.), or the like.

Identification of partition populations may include assigning each partition to one of a plurality of predefined bins each corresponding to a distinct partition population.

The predefined bins may represent all possible combinations of positives for the target, or only a subset of the all possible combinations (e.g., where N≥3, all combinations of positives for zero, one, and two targets). Further aspects of population identification are presented below in Examples 6 and 7.

Obtain partition counts. A partition count for each partition population may be obtained, indicated at **52**. The partition count may be a value representing the number of partitions constituting a particular partition population.

A number of partitions that are positive (or negative) for each target may be determined for the signal, indicated at **50**. The signal detected from each partition, and the partition itself, may be classified as being positive or negative for each of the reactions/targets contributing to the signal. Classification may be based on the strength (and/or other suitable aspect) of the signal. If the signal/partition is classified as positive (+), for a given target, the reaction corresponding to that target is deemed to have occurred and at least one copy of the target is deemed to be present in the partition. In contrast, if the signal/partition is classified as negative (−), for a given target, the reaction corresponding to that target is deemed not to have occurred and no copy of the target is deemed to be present in the partition (i.e., the target is deemed to be absent from the partition). The data including all permutations of positives will generally fall into $2^N$ populations or clusters, where N is the number of targets, assuming that each population is distinguishable. Exemplary results for one, two, and three target systems in which data are collected in a single channel are shown in the following tables:

| | Target A | Intensity |
|---|---|---|
| Population 2 | + | Highest |
| Population 1 | − | Lowest |

| | Target A | Target B | Intensity |
|---|---|---|---|
| Population 4 | + | + | Highest |
| Population 3 | + | − | Intermediate |
| Population 2 | − | + | Intermediate |
| Population 1 | − | − | Lowest |

| | Target A | Target B | Target C | Intensity |
|---|---|---|---|---|
| Population 8 | + | + | + | Highest |
| Population 7 | + | + | − | Intermediate |
| Population 6 | + | − | + | Intermediate |
| Population 5 | − | + | + | Intermediate |
| Population 4 | + | − | − | Intermediate |
| Population 3 | − | + | − | Intermediate |
| Population 2 | − | − | + | Intermediate |
| Population 1 | − | − | − | Lowest |

Determination of target levels. Levels of all of the targets may be determined, indicated at **54**, based on various combinations of the counts obtained. Determination of target levels may (or may not) be based on each target having a Poisson distribution among the partitions. Each level may, for example, be a value representing the total number of partitions positive for the target, or a concentration value, such as a value representing the average number copies of the target per partition. The partition data further may be used (e.g., directly and/or as concentration data) to estimate copy number (CN) and copy number variation (CNV), using

any suitable algorithms such as those described below and elsewhere in the present disclosure.

A level (e.g., concentration) of each target may be determined with Poisson statistics. The concentration may be expressed with respect to the partitions and/or with respect to a sample providing the target. The concentration of the target in the partitions may be calculated from the fraction of positive partitions (or, equivalently, the fraction of negative partitions) by assuming that copies of the target (before amplification) have a Poisson distribution among the partitions. With this assumption, the fraction f(k) of partitions having k copies of the template is given by the following equation:

$$f(k) = \frac{C^k}{k!}e^{-C} \quad (1)$$

Here, C is the concentration of the target in the partitions, expressed as the average number of target copies per partition (before amplification). Simplified Poisson equations may be derived from the more general equation above and may be used to determine target concentration from the fraction of positive partitions. An exemplary Poisson equation that may be used is as follows:

$$C = -\ln\left(1 - \frac{N_+}{N_{tot}}\right) \quad (2)$$

where $N_+$ is the total number of partitions (i.e., the partition count) positive for a given target, and where $N_{tot}$ is the total number of partitions that are positive or negative for the target. $N_{tot}$ is equal to a sum of (a) $N_+$ for the target and (b) the number of partitions negative for the target, or $N_-$. $N_+/N_{tot}$ (or $N_+/(N_++N_-)$ is equal to $f_+$, which is the fraction of partitions positive for the template (i.e., $f_+=f(1)+f(2)+f(3)+\ldots$) (see Equation (1)), and which is a measured estimate of the probability of a partition having at least one copy of the template. Another exemplary Poisson equation that may be used is as follows:

$$C = -\ln\left(\frac{N_-}{N_{tot}}\right) \quad (3)$$

where $N_-$ and $N_{tot}$ are as defined above. $N_-/N_{tot}$ is equal to $f_-$, which is the fraction of negative partitions (or $1-f_+$), is a measured estimate of the probability of a partition having no copies of the target, and C is the target concentration as described above.

Equations (2) and (3) above can be rearranged to produce the following:

$$C=\ln(N_{tot})-\ln(N_{tot}-N_+) \quad (4)$$

$$C=\ln(N_{tot})-\ln(N_-) \quad (5)$$

The concentration of each target in a multiplexed assay can, for example, be determined with any of Equations (2)-(5), using values (i.e., partition counts) obtained for $N_{tot}$ and $N_-$ or $N_+$, for each target. In some cases, the value used for $N_{tot}$ (the total partition count) may be the same for each target. In other cases, the value used for $N_{tot}$ may vary, such as if some of the populations are excluded from the total count due to population overlap. In some embodiments, $N_{tot}$ may be equivalent to a combination of all populations, namely, a sum of the partition counts for all populations identified.

The value used for $N_-$ or $N_+$ is generally different for each target, and may result from summing the counts from a plurality of partition populations each containing a different combination of the targets being tested in the multiplexed assay. For example, with three targets (A, B, and C) in a multiplexed assay, the number of partitions positive for target A, $N_{+A}$, may be calculated as the sum of counts from the single (A only), double (AB and AC), and triple (ABC) positive populations, for use in Equation (2) or (4). Equivalently, the number of partitions negative for target A, $N_A$, may be calculated, for use in Equation (3) or (5), as the difference between $N_{tot}$ and $N_{+A}$. Alternatively, the number partitions negative for A may be calculated as the sum of counts from each population that is negative for target A, namely, in this example, a triple negative ("empty") population, two single positive populations (B and C), and one double positive population (BC). The same process may be repeated for each of the other targets using partition counts from the appropriate subset of populations. In any event, determination of at least one target concentration and/or each target concentration may be based on a combination of counts from at least two populations and with the exclusion of at least two populations. In some cases, a combination of counts used for at least one target or for each target may include counts from only one-half of the populations.

In some embodiments, an estimate of the level of the template may be obtained directly from the positive fraction, without use of Poisson statistics. In particular, the positive fraction and the concentration (copies per partition) converge as the concentration decreases. For example, with a positive fraction of 0.1, the concentration is determined with Equation (2) to be about 0.105, a difference of only 5%; with a positive fraction of 0.01, the concentration is determined to be about 0.01005, a ten-fold smaller difference of only 0.5%. However, the use of Poisson statistics can provide a more accurate estimate of concentration, particularly with a relatively higher positive fraction, because the equation accounts for the occurrence of multiple target copies per partition.

Further aspects of sample preparation, partition formation, data collection, population identification, obtaining partition counts, and target level determination, among others, that may be suitable for the system of the present disclosure are described elsewhere in the present disclosure, such as below in Examples 5-7, and in the references identified above in the Cross-References, which are incorporated herein by reference.

FIG. **2** shows an exemplary system **60** for performing the digital assay of FIG. **1**. System **60** may include a partitioning assembly, such as a droplet generator **62** ("DG"), a thermal incubation assembly, such as a thermocycler **64** ("TC"), a detection assembly (a detector) **66** ("DET"), and a data processing assembly (a data processor) **68** ("PROC"), or any combination thereof, among others. The data processing assembly may be, or may be included in, a controller that communicates with and controls operation of any suitable combination of the assemblies. The arrows between the assemblies indicate movement or transfer of material, such as fluid (e.g., a continuous phase of an emulsion) and/or partitions (e.g., droplets) or signals/data, between the assemblies. Any suitable combination of the assemblies may be operatively connected to one another, and/or one or more of

the assemblies may be unconnected to the other assemblies, such that, for example, material/data are transferred manually.

Detector **66** may provide a plurality of optical channels in which data can be collected. The detector may have a distinct sensor or detection unit for each optical channel.

System **60** may operate as follows. Droplet generator **62** may form droplets disposed in a continuous phase. The droplets may be cycled thermally with thermocycler **64** to promote amplification of targets in the droplets. Signals may be detected from the droplets with detector **66**. The signals may be processed by processor **68** to determine numbers of droplets and/or target levels, among others

II. Examples

This section presents selected aspects and embodiments of the present disclosure related to methods of performing multiplexed digital assays.

### Example 1

### Digital PCR Assays with Multiplexed Detection in the Same Channel

This example describes an exemplary digital PCR assay with multiplexed detection of two targets, using two probes, analyzed in the same channel. Other assays may involve three or more targets and three or more probes, where at least two targets are analyzed in the same channel.

FIG. **3** shows a pair of targets **80**, **82** ("Target 1" and "Target 2") and corresponding probes **84**, **86** ("Probe 1" and "Probe 2") that may be used to create a dedicated signal for each target in a digital PCR assay. Each probe may include an oligonucleotide **88**, **90**, a fluorophore **92**, **94**, and a quencher **96**. Each of the fluorophore and the quencher may (or may not) be conjugated to the oligonucleotide by a covalent bond. The probe also or alternatively may include a binding moiety (a minor groove binder) for the minor groove of a DNA duplex, which may be conjugated to the oligonucleotide and which may function to permit a shorter oligonucleotide to be used in the probe.

Each oligonucleotide may provide target specificity by hybridization predominantly or at least substantially exclusively to only one of the two targets. Hybridization of the oligonucleotide to its corresponding target is illustrated schematically at **98**.

Fluorophores **92**, **94**, which may be the same or different, create detectable but distinguishable signals in the same channel, allowing multiplexing in that channel. The signals may be distinguishable because an aspect of the fluorescence is different for one fluorophore than for the other fluorophore(s). For example, the intensity associated with one fluorophore, following reaction, may be lower or higher than the intensity(ies) associated with the other fluorophore(s). In some embodiments, one probe may be labeled with a different number of fluorophores than the other probe, and/or the probes may be located in slightly different local environments, creating a different level of fluorescence for each probe following reaction. Alternatively, or in addition, both probes may be labeled with the same number of fluorophores (e.g., one fluorophore), but there may be more or less of one probe than the other in the sample, so that a greater or smaller signal is created when the reactions have occurred. In some cases, the fluorophores themselves might be different, with one more or less intrinsically fluorescent than the other (e.g., due to differences in extinction coefficient, quantum yield, etc.), so long as each fluorophore can be detected in the same channel. Exemplary fluorophores that may be suitable include FAM, VIC, ROX, TAMRA, JOE, etc., among others.

Quencher **96** is configured to quench the signal produced by fluorophore **92** or **94** in a proximity-dependent fashion. Accordingly, light detected from the fluorophore may increase when the associated oligonucleotide **88** or **90** binds to the amplified target, to increase the separation between the fluorophore and the quencher, or when the probe is cleaved and the fluorophore and quencher become uncoupled during target amplification, among others. The quencher may be the same or different for each type of fluorophore. Here, the assay is designed so that the presence of a target gene leads to an increase in corresponding intensity, because amplification reduces quenching. In other assays, the reverse could be true, such that the presence of a target caused a decrease in corresponding intensity (although it typically is easier to detect a signal against a dark background than the opposite). Moreover, some embodiments may be constructed without a quencher, so long as the fluorescence and so the signal changes upon amplification.

FIG. **4** shows an exemplary graph **102** of data corresponding to an exemplary digital PCR assay for Target 1 and Target 2 performed in droplets. The graph plots a signal **104** that represents light detected from probes **84**, **86** (and/or one or more modified (e.g., cleavage) products thereof) (see FIG. **3**). The signal is created from light detected over time in a single channel from a fluid stream containing the droplets and flowing through an examination region of the channel. The signal may be analyzed to determine whether neither Target, Target 1 alone, Target 2 alone, or both Targets 1 and 2 are present in each droplet. In particular, the strength or intensity of the signal in a system with two targets may be divided or thresholded into four intervals corresponding to no Target (Interval 1), Target 1 alone (Interval 2), Target 2 alone (Interval 3), or both Targets 1 and 2 (Interval 4):

- Peaks **106** with maxima in Interval 1 correspond to droplets containing no Target (T1−/T2−). The measured signal corresponds to background (e.g., background fluorescence, scattering, etc.) and does not reflect the presence or amplification of either Target.
- Peaks **108** with maxima in Interval 2 correspond to droplets containing Target 1 but not containing Target 2 (T1+/T2−). The measured signal corresponds to signal from Target 1 plus background and reflects amplification of Target 1 implying the presence of Target 1.
- Peaks **110** with maxima in Interval 3 correspond to droplets containing Target 2 but not containing Target 1 (T1−/T2+). The measured signal corresponds to signal from Target 2 plus background and reflects amplification of Target 2 implying the presence of Target 2.
- Peaks **112** with maxima in Interval 4 correspond to droplets containing both Targets 1 and 2 (T1+/T2+). The measured signal corresponds to signal from both Targets 1 and 2 plus background and reflects amplification of Targets 1 and 2 implying the presence of Targets 1 and 2.

In the present example, each droplet, whether positive or negative for each target, produces an increase in signal strength above the baseline signal that forms an identifiable peak **106**, **108**, **110**, **112**. Accordingly, the signal may vary in strength with the presence or absence of a droplet and with the presence or absence of a corresponding target.

The assignment of a droplet to a particular outcome (i.e., to one of T1−/T2−, T1+/T2−, T1−/T2+, and T1+/T2+) may be performed using any suitable algorithm. In the example above, peak heights (i.e., intensity values) associated with each outcome are sufficiently different that each can be

unambiguously identified and assigned. Specifically, the peaks are assigned based on intervals delineated by values lying between (e.g., half way between) the peak heights for one outcome and the peak heights for adjacent outcomes. In other cases, the peak heights for each outcome may overlap at their extremes, so that thresholding may be neither simple nor linear. In such cases, statistical methods such as expectation maximization algorithms may be used to estimate the number of droplets or peaks associated with each outcome and the associated concentrations.

## Example 2

### Digital PCR Assay to Assess Copy Number of C61

This example describes a first exemplary digital PCR assay, in which multiplexing in a single channel is used to assess copy number of the C61 gene; see FIGS. **5** and **6**. Specifically, signals from two probes, one for the gene of interest, C61, and one for a reference gene, RPP30, are collected together, as a single signal, in a single channel and used to assess the number of copies of the gene of interest relative to the number of copies of the reference gene.

The principles described here may be used with any suitable gene(s). In this example, C61 is a gene of interest, for which information on copy number is sought, and RPP30 is a reference gene, which codes for ribonuclease P protein subunit p30, that is known to have two copies per genome.

The principles described here also may be extended to additional genes of interest, for example, two or three or more genes of interest, and may or may not involve reference genes such as RPP30. The number of copies may be determined absolutely, if the copy number of at least one of the genes (e.g., the reference gene) is known, or relatively, if the copy number of none of the genes is known.

FIG. **5** is a scatter plot showing data for the exemplary C61/RPP30 system. Specifically, FIG. **5** shows intensity in the FAM channel plotted as a function of intensity in the VIC channel for each droplet in a digital PCR assay. (Here and elsewhere in the present disclosure, the dots of a scatter plot represent individual droplets.) Visually, the data comprise four distinct populations, corresponding to four distinct ranges of FAM intensity (the intensities in the VIC channel are all low and overlapping). The assay is constructed so that amplification of C61 leads to a lower FAM intensity than amplification of RPP30 (although it would work as well if the reverse were true). The four populations may be summarized as follows:

- Population 1, with the lowest FAM intensity, corresponds to droplets that are negative for RPP30 and C61 (i.e., droplets that did not include either gene).
- Population 2, with the lower of two intermediate FAM intensities, corresponds to droplets that are positive for C61 and negative for RPP30 (i.e., droplets that included the C61 gene but did not include the RPP30 gene).
- Population 3, with the higher of two intermediate FAM intensities, corresponds to droplets that are positive to RPP30 and negative for C61 (i.e., droplets that included the RPP30 gene but did not include the C61 gene).
- Population 4, with the highest FAM intensity, corresponds to droplets that are positive for RPP30 and C61 (i.e., droplets that included both genes).

The number of droplets in each population may be counted using any suitable mechanism(s), during or following data acquisition. Here, because the intensities are widely separated, the number may be counted by assigning suitable intensity ranges or intervals to each population, as in Example 1, so that droplets falling within a selected intensity range are designated as falling within the population corresponding to that range. The results of such counting are summarized in the following table:

| | RPP30 | C61 | # Droplets |
|---|---|---|---|
| Population 4 | + | + | 1168 |
| Population 3 | + | – | 2865 |
| Population 2 | – | + | 2854 |
| Population 1 | – | – | 6782 |

Here, + means that the assay is positive for the indicated gene (i.e., that the indicated gene is present), and – means that the assay is negative for the indicated gene (i.e., that the indicated gene was absent). There are 4033 droplets containing RPP30 (i.e., that are positive for RPP30, irrespective of whether they are positive or negative for C61), as determined by adding the number of droplets in Populations 3 and 4 (i.e., by adding 2865 and 1168, respectively). There are 4022 droplets containing C61 (i.e., that are positive for C61, irrespective of whether they are positive or negative for RPP30), as determined by adding the number of droplets in Populations 2 and 4 (i.e., by adding 2854 and 1168, respectively). Thus, the ratio of C61 to RPP30 is 4022/4033=0.997=1:1 within experimental error. Thus, because RPP30 is known to have two copies per genome, C61 must also have two copies (i.e., the copy number of C61 is two). In many cases, concentrations of the targets are determined by Poisson statistics (e.g., using one or more of Equations (2)-(5)), before a ratio of target levels is determined.

FIG. **6** shows FAM intensities for the C61/RPP30 system of FIG. **5** for twelve different sets of droplets exposed to twelve different annealing temperatures (denoted, from left to right, 55.0, 55.2, . . . , 64.6, and 64.9) during thermal cycling to promote target amplification. The data show that there is sufficient resolution between the four populations to perform the assay in a single channel under a variety of experimental conditions. The data shown in FIG. **5** correspond to one of the conditions in this plot, namely, amplification with an annealing temperature of 59.1° C. (marked in FIG. **6** with an arrowhead).

The annealing temperature may be selected from among the various temperatures tested, based on comparison of collected intensity data. For example, the resolution or separation of each population of droplets from one another in the plot for the various annealing temperatures may be compared to permit selection of a suitable annealing temperature for further data collection and/or analysis. For example, here, the annealing temperature of 59.1° C. offers the best separation between each different population of droplets within the set. In particular, at this annealing temperature, data from double-positive droplets (RPP30+/C61+; the population of highest intensity) are well resolved from data for single-positive droplets (RPP30+ or C61+; the two populations of intermediate intensity), which in turn are well resolved from each other and from data for double-negative droplets (RPP30–/C61–; the population of lowest intensity).

The resolution provided by selection of an optimal annealing temperature may permit determination, for each target, a respective number of droplets that are positive for the target alone at the selected annealing temperature. Also, at least one number of droplets positive for more than one

target may be determined. The total number of droplets positive for each target then may be determined based on the respective numbers and the at least one number.

In some cases, signal detection may be performed first on only a fraction of each set of droplets. Additional droplets from the particular set corresponding to the selected annealing temperature then may be run through the detector to provide additional data for analysis. In other cases, only a fraction of the data collected for each set of droplets may be plotted and/or compared, and then additional data collected for the particular set corresponding to the selected annealing temperature may be plotted and/or analyzed.

## Example 3

### Digital PCR Assay to Assess Copy Number of C63

This example describes a second exemplary digital PCR assay, in which multiplexing in a single channel is used to assess copy number of the C63 gene; see FIGS. **7** and **8**. Specifically, signals from two probes, one for the gene of interest, C63, and one for a reference gene, again RPP30, are collected in a single channel and used to assess the number of copies of the gene of interest relative to the number of copies of the reference gene.

FIG. **7** is a graph showing data for the exemplary C63/RPP30 system. Like FIG. **5**, FIG. **7** shows intensity in the FAM channel plotted as a function of intensity in the VIC channel for each droplet in a digital PCR assay. However, unlike FIG. **5**, this system includes probes for RPP30 and C63 instead of RPP30 and C61. The data again show four distinct populations, corresponding in order of decreasing intensity in the FAM channel to RPP30+/C63+, RPP30+/C63−, RPP30−/C63+, and RPP30−/C63−. The number of droplets counted in each of these bins corresponds to 3552 RPP30 positive droplets, 3441 C63 positive droplets, and 6224 negative droplets. Thus, the ratio of C63 to RPP30 is 3441/3552=0.969=1:1 within experimental error. Thus, like C61, C63 must have two copies (i.e., the copy number of C63 is two).

FIG. **8**A shows FAM intensities and FIG. **8**B shows VIC intensities for the C63/RPP30 system of FIG. **7** for twelve different sets of experimental conditions (i.e., different annealing temperatures for amplification). The data show that there is sufficient resolution between the four populations to perform the assay in a single channel under a variety of experimental conditions. The data shown in FIG. **7** correspond to one of the conditions in these plots.

## Example 4

### Digital Assay with More Luminophores than Optical Channels

This example describes an exemplary digital assay with multiplexed analysis of targets using more luminophores than the number of optical channels used for detecting signals from the luminophores; see FIGS. **9** and **10**.

FIG. **9** shows an exemplary bulk phase mixture **140** being separated into droplets during performance of an exemplary digital assay with multiplexed detection of three or more distinct targets (e.g., identified here as T1, T2, and T3). Mixture **140** may include a forward primer **142** and a reverse primer **144** for each target. The mixture also may include a reporter for amplification of each target (such as a T1 probe, a T2 probe, and a T3 probe). Each reporter may be a probe including an oligonucleotide labeled with a different luminophore (namely, L1, L2, and L3, respectively). Accordingly, luminescence from the T1-T3 probes, and more particularly, the L1, L2, and L3 luminophores, may report whether or not targets T1, T2, and T3, respectively, are present in each individual droplet. Each probe also may include a quencher for the associated luminophore.

FIG. **10** schematically represents an exemplary scatter plot showing droplet (or peak) intensities for a T1/T2/T3 droplet assay that may be performed with the droplets of FIG. **9**. Data from the T1, T2, and T3 probes are collected in a first optical channel and a second optical channel and plotted as a function of intensity in the first channel (vertical axis) versus intensity in second channel (horizontal axis). Intensities produced by exemplary populations or clusters of negative, T1-positive, T2-positive, and T3-positive droplets are identified by filled ellipses, with the size of each ellipse corresponding to the number of droplets in each population. To simplify the presentation, double-positive populations (T1+/T2+, T1+/T3+, and T2+/T3+) and the triple-positive population (T1+/T2+/T3+) are not shown. (However, see Example 7 for an exemplary distribution of single- and multiple-positive droplets.)

Each luminophore may be at least predominantly or substantially exclusively detectable in only one optical channel, or may be substantially detectable in two or more optical channels. For example, L1 of the T1 probe is substantially detectable in the first channel but not the second channel, L2 of the T2 probe is substantially detectable in the second channel but not the first channel, and L3 of the T3 probe is substantially detectable in both channels. More generally, L3 may generate a distinguishable intensity and/or a different ratio of signal intensities for amplification of the T3 target in each channel relative to the intensity and/or ratio produced by L1 for amplification of the T1 target and produced by L2 for amplification of the T2 target. As a result, the population of T3-only positives is resolved from the T1-only and T2-only populations. Also, the populations containing more than one target may be resolved and distinguishable from one another and from the single-target populations and the negative population (e.g., see Example 7).

Any suitable number of targets may be analyzed with the approach presented in this Example. For example, R targets may be analyzed using R distinct luminophores and less than R optical channels for detection of target amplification.

## Example 5

### Adjustment of Individual Assay Concentrations in a Multiplexed Assay

This example describes an exemplary approach for adjusting the endpoint intensity of individual target assays of a multiplexed assay; see FIG. **11**.

Digital PCR enables accurate, precise, and sensitive quantification of specific nucleic acid sequences. In addition to the detection of two targets using two different fluorophores and two optical channels for collecting data from the fluorophores, it is possible to increase the number of targets detected by varying parameters that affect PCR efficiency and thus the level of endpoint fluorescence for each target. The present disclosure describes a method to multiplex assays by adjusting the endpoint fluorescence level (and/or spectrum) for each target assay (e.g., by changing the concentration of primers and/or probes and/or the type of fluorophores used). This approach allows users to expand the number of simultaneously detected targets without increasing the number of optical channels for detection.

Increasing the number of potential targets per test is a significant improvement for digital PCR, augmenting dramatically the information output of each sample.

FIG. **11** shows a graph of fluorescence intensity data obtained in a one-target amplification assay performed in droplets. Here, fluorescence is measured with a single detector (one optical channel) as droplets flow past the detector in a stream of carrier fluid. Droplets or "events" may be identified as periodic spikes or waves in the fluorescence signal (e.g., see FIG. **4** above). Each droplet/event then may be assigned at least one fluorescence value, e.g., representing an amplitude and/or integrated intensity, among others, of the corresponding signal wave. The fluorescence intensity value (in arbitrary units ("arb.")) for each droplet then may be plotted as a function of event number. At the left side of the graph ("stock"), droplets that are amplification-negative and amplification-positive form well-resolved bands of lower and higher intensity, respectively.

The assay was performed with a series of two-fold dilutions of assay reagents, namely, the primers and the probe, as indicated above the graph. Changing the concentration of one or more assay reagents, other than the template, may be described as changing the assay concentration. Each successive dilution of the assay (primers and probe), up to eight-fold, markedly decreased the level (interchangeably termed amplitude) of the fluorescence endpoint for the target-positive droplets (indicated by arrows), and more subtly decreased the intensity of the target-negative droplets. However, droplet counts from each successive dilution up to eight-fold provided a reproducible target concentration (presented as "copies/μL"). Therefore, changing the fluorescence endpoint for a target can be achieved without degrading the ability to accurately and reliably determine the target concentration. The probe and primers for an assay each may be adjusted by the same factor, as shown in FIG. **11**, or each may be adjusted by a different factor (or not at all).

The present disclosure provides a method to assay for multiple targets (multiplex) through digital PCR, by varying the assay used for each target. An assay may include a set of primers (forward and reverse) and a fluorescently labeled probe. In digital PCR, droplets may be segregated by the levels of fluorescence produced by the PCR reaction. The level of fluorescence for a droplet is dependent on a multitude of factors (reaction efficiency, type of fluorophores, amplicon size, etc.). By changing the concentration of an assay, the reaction efficiency of a particular target can be affected, which may result in a difference in fluorescence level that allows populations detected with the same fluorophore to be distinguished from one another. By changing the assay concentrations of two different fluorophores, additional targets may be detected in the same multiplexed reaction. In some cases, fluorescence intensity may be adjusted by varying the concentration of one or both primers for a target. Varying primer concentration without changing the probe concentration may be useful in assays where the same probe is used to detect two targets, but each of the two targets is amplified with at least one different primer. In some cases, the level of the fluorescence for a target may be adjusted by changing the annealing temperature used for thermocycling, the total concentration of dNTPs, the amounts of individual dNTPs relative to each other (e.g., if the two targets have substantially different base compositions), or the like.

## Example 6

### Multiplexed Assays with Orthogonal Droplet Populations

This example describes exemplary multiplexed digital assays performed on R targets, where R is at least three, and with fewer than R optical channels, where amplification of each target is substantially detectable in only one of the optical channels (e.g., two optical channels). As a result, droplet populations positive for different combinations of the targets are arranged in rows and columns in a plot of detected amplification data; see FIGS. **12-18**.

FIG. **12** is a 2-D scatter plot of fluorescence intensity data obtained from individual droplets detected in two optical channels during performance of a triplex amplification assay for three targets (RPP30, Chr10q1, and Chr13q3). Each dot represents a single droplet. Before droplet formation, the template DNA was digested with AluI, which reduces the size of the template DNA but does not cut between the priming sites for any of the three targets. Amplification of RPP30 was detected with a FAM-labeled probe in optical channel one, and amplification of chromosome 10 and chromosome 13 regions (10q1 and 13q3) was detected with VIC-labeled probes in optical channel two, at a different waveband of light emission than channel one. The three assays (RPP30, Chr10q1, and Chr13q3) were respectively run at probe/primer concentrations of 1×, 1×, and 0.6×. Accordingly, the chromosome 13q3 assay produced a lower fluorescence endpoint signal than the chromosome 10q1 assay because the 13q3 assay was more dilute (0.6× versus 1×).

Eight droplet populations are visible and resolved from each other in FIG. **12**, with the droplet populations separated from one another by dashed lines. The populations are as follows: one triple negative ("empty"), three single positives (each positive for RPP30, 10q1, or 13q3), three double positives (positive for each pair-wise combination of the targets), and one triple positive (positive for all three targets). The concentrations of the targets can be calculated based on combination of counts from the populations. For example, the concentration of 10q1 can be calculated using Equation (4) and a positive droplet count ($N_+$) representing a combination of the four 10q1-positive populations (and excluding the four 10q1-negative populations), or with Equation (5) using a negative droplet count ($N_-$) representing a combination of only the four 10q1-negative populations (and excluding the four 10q1-positive populations). In any event, counts from a plurality of 10q1-positive populations (e.g., one-half of all populations identified) are excluded.

FIG. **13** shows another 2-D scatter plot of fluorescence intensity data obtained generally as in FIG. **12** but with the sample (template DNA) digested before amplification with a restriction enzyme (HaeIII) that cuts between the priming sites of the Chr10q1 template. As a result, the droplet counts for each of the four 10q1-positive populations decreases dramatically, while the droplet counts for each of the four 10q1-negative populations increases, thereby providing validation for the assignment of droplet populations. Also, the experiment demonstrates that the multiplexed assay system can produce an additive effect of the fluorescence levels from two different assays using the same fluorophore for both probes.

FIG. **14** shows a 2-D scatter plot of fluorescence intensity data obtained as in FIG. **12**, but with the concentrations of the Chr13q3 and Chr10q1 assays adjusted to change the endpoint fluorescence level for assay of these targets. In particular, the 10q1 assay is performed at a 0.6× concentration (instead of 1×) and the 13q3 assay at a 1× concentration (instead of 0.6×). Accordingly, the positions of droplet populations are rearranged with respect to FIG. **12**, with the 10q1-positive populations that are negative for 13q3 having a lower intensity than the 13q3-positive populations that

negative for 10q1. As in FIG. **12**, the concentration of all three targets is equivalent (i.e., the sample is from a euploid genome).

FIG. **15** shows another 2-D scatter plot of fluorescence intensity data obtained with the assay concentrations of FIG. **14**, but with the sample (template DNA) digested before amplification with a restriction enzyme (HaeIII) that cuts between the priming sites of the Chr10q1 template. The digestion produces a significant reduction of populations containing Chr10q1-positive droplets, therefore validating the detection pattern.

FIG. **16** shows a 2-D scatter plot of fluorescence intensity data obtained from individual droplets detected in two optical channels during performance of a tetraplex amplification assay for four targets (RPP30, MP10K, Chr13q3, and Chr10q1). RPP30 and MP10K targets were detected with FAM-labeled probes using 1× and 1.5× assay concentrations, respectively. Chr13q3 and Chr10q1 targets were detected with VIC-labeled probes using 0.6× and 1× assay concentrations, respectively. The resulting droplet populations are separated by dashed lines in the plot, and the target composition of each droplet population is indicated ("−" is negative and "+" is positive for each indicated target). The assay provides simultaneous detection of four different targets in two optical channels. The results illustrate the capacity of the multiplexed assay system to separate discrete populations by levels of fluorescence using the same fluorophores.

The droplet populations have an orthogonal arrangement in a scatter plot with a linear scale for each axis. Stated differently, the droplet populations may form a matrix or rectangular array, which may have $2^R$ populations, where R is the number of targets. The populations may form an array having a plurality of rows and a plurality of columns each containing two or more populations. Each row may contain the same number of populations, and each column may contain the same number of populations. In some embodiments, each of the single-positive populations may be restricted to an edge of the array, such as the left-most column or the bottom row of the array. With this arrangement, multiply-positive populations can be distributed in the optical space above the bottom row and to the right of the left-most column, without overlapping one another or any single-positive populations. Also, the positions of all of the multiply-positive populations can be predicted from the positions of the single-positive populations, simplifying the task of selecting suitable intensities for each single-positive population during assay development and optimization. To achieve this arrangement of single-positive populations, each target may have a corresponding probe that is detectable substantially exclusively in only one of the optical channels, such that amplification of each target is detected substantially in only one of the optical channels. For example, amplification of RPP30 and MP10K (FAM-labeled probes) is detectable substantially exclusively in channel one, and amplification of 13q3 and 10q1 (VIC-labeled probes) substantially exclusively in channel two.

FIG. **17** shows the 2-D scatter plot of FIG. **16**, with the dashed lines removed and each droplet population circled using a line weight that indicates the number of different targets for which each population is amplification positive. The plot has one quadruple-negative population (at the bottom left corner of the population array), four single-positive populations ("1+"), six double-positive populations ("2+"), four triple-positive populations (3+"), and one quadruple-positive population ("4+").

FIG. **18** shows a 2-D scatter plot of fluorescence intensity data obtained with the tetraplex assay of FIG. **16**, but with the sample (template DNA) diluted by a factor of four relative to FIG. **16**. The plot exhibits a visible reduction of droplets from positive populations, particularly the triple- and quadruple-positive populations. The plot demonstrates that the multiplexed assay system retains its ability to quantify targets in a multiplex setting.

## Example 7

### Multiplexed Assays with Non-orthogonal Droplet Populations

This example describes exemplary multiplexed digital assays performed on R targets, where R is at least three, and with fewer than R optical channels (e.g., only two optical channels), where amplification of at least one, two or more, or each of the targets is substantially detectable in two or more of the optical channels. As a result, droplet populations positive for a plurality of combinations of the targets are arranged non-rectangularly, with less apparent order, in a plot of detected amplification data; see FIGS. **19-25**.

FIG. **19** shows a 2-D scatter plot of fluorescence intensity data obtained from individual droplets detected in two optical channels during performance of a tetraplex amplification assay for four targets (Chr10q1, Chr13q3, Chr16p1, and ChrXq3). Each dot represents a single droplet. Amplification of the four targets was detected using four corresponding probes each containing a different fluorophore (Alexa® 488 dye, Atto® 488 dye, VIC dye, and TET dye, respectively), as labeled in the plot. Each resolved dot represents a single droplet. In this experiment, multiplexing was achieved by using "non-traditional" fluorophores (i.e., other than FAM and VIC/HEX), although traditional and non-traditional fluorophores may be used together in a multiplexed assay. Each fluorophore may be attached to a distinct oligonucleotide. Each fluorophore may (or may not) exhibit substantial emission in each optical channel or may be substantially detectable in only one of the optical channels. In the present example, the four fluorophores have distinct spectral profiles, with each detectably emitting light in both optical channels. As a consequence, the droplet populations are arranged in a non-orthogonal pattern that allows differentiation of more targets than the number of optical channels.

FIGS. **20-23** each reproduce the scatter plot of FIG. **19** and respectively show a circle around each single- ("1+"), double- ("2+"), triple- ("3+"), and quadruple ("4+")-droplet population. Arrows indicate the distinct target composition of each population. The assignment of droplet populations was confirmed by performing four different triplex reactions in which each of the different assays (for each target) was separately omitted from the reaction mixture.

FIG. **24** shows a composite of FIGS. **20-23** that marks all of the droplet populations in the same plot. The approach allows simultaneous identification of all droplet populations.

FIG. **25** is a 2-D scatter plot obtained as in FIGS. **19** and **24** but with only one-sixth the amount of template DNA. The size of each positive population is reduced, with a substantially greater effect on the size of the triple- and- quadruple positive populations. The result illustrates that the multiplexed assay system retains its ability to quantify targets when using combinations of non-traditional fluorophores.

The multiplexed approaches disclosed here permit quantification of more targets that the number of optical channels. Examples 6 and 7 utilize only two optical channels. How-

ever, the multiplexed assay system of the present disclosure may collect data in any suitable number of C optical channels, to collect data in a C-dimensional optical space. The data may be processed in a manner analogous to that described above for a two-dimensional optical space.

Example 8

Selected Embodiments

This example describes selected embodiments of exemplary multiplexed digital assays, presented as a series of numbered paragraphs.

1. A method of performing a multiplexed digital assay, comprising: (A) forming droplets that collectively contain R targets; (B) amplifying the targets in the droplets; (C) collecting data representing amplification of the targets in the droplets, the data being collected in fewer than R optical channels; and (D) determining a level of each target with the data, wherein the level determined for at least one of the targets is based in part on a droplet count for a droplet population identified as positive for two of the targets.

2. The method of paragraph 1, wherein R is at least three.

3. The method of paragraph 1 or 2, wherein the data is collected in only two optical channels.

4. The method of paragraph 1, wherein each optical channel represents a different waveband of emitted light.

5. The method of any preceding paragraph, wherein each optical channel represents a different waveband of excitation light.

6. The method of paragraph 1, wherein a different plurality of the droplets contains a copy of each pair of the targets.

7. The method of paragraph 1, wherein at least about 5%, 10%, 20%, or 50% of the droplets contain a pair of the targets.

8. The method of paragraph 1, further comprising a step of identifying from the data a plurality of droplet populations each positive for a different combination of the targets.

9. The method of paragraph 8, wherein forming, amplifying, and collecting are performed such that each droplet population that is positive for exactly two of the targets is at least substantially resolved from each droplet population that is positive for only one of the targets.

10. The method of paragraph 1, further comprising a step of plotting the data such that droplet populations containing different combinations of the targets form a rectangular array.

11. The method of paragraph 10, wherein the array is composed of a plurality of rows and columns, with each row and each column including at least two of the droplet populations.

12. The method of paragraph 10, wherein the step of plotting is performed with respect to a pair of orthogonal axes, and wherein each droplet population that is positive for only one of the targets is arranged along a line that is at least generally parallel to one of the axes.

13. A method of performing a multiplexed digital assay, comprising: (A) forming droplets collectively containing R targets, where R is at least three, and where a significant number of the droplets contain a copy of each of two or more of the targets; (B) amplifying the targets in the droplets; (C) collecting data representing amplification the targets in the droplets, the data being collected in fewer than R optical channels each representing a distinct waveband of emitted light; (D) identifying from the data a plurality of droplet populations, wherein forming, amplifying, and collecting are performed such that each droplet population positive for two of the targets is at least substantially resolved from each droplet population positive for only one of the targets; and (E) determining a level of each target based in part on one or more droplet populations identified as containing a pair of the targets.

14. A method of performing a multiplexed digital assay, comprising: (A) amplifying R targets in droplets; (B) collecting data representing amplification of the targets in the droplets, the data being collected in a plurality of less than R optical channels each representing a distinct waveband of light; (C) identifying from the data a plurality of droplet populations each containing a different combination of the targets; and (D) determining a level of each target, wherein the step of determining for each target is based on a value for droplet counts obtained from a combination of at least two droplet populations that excludes at least two other droplet populations.

15. The method of paragraph 14, wherein one of the plurality of droplet populations contains none of the targets.

16. The method of paragraph 14, wherein one of the plurality of droplet populations contains a copy of each of the targets.

17. A method of performing a digital assay, comprising: (A) forming droplets that collectively contain R targets and a reporter corresponding to each target, wherein R is at least three and each reporter includes a different luminophore; (B) amplifying the targets in the droplets; (C) collecting data representing amplification of the targets in the droplets, the data being collected in less than R optical channels each representing a distinct waveband of light; and (D) determining a level of each target based on the data.

18. The method of paragraph 17, wherein at least one of the reporters reports amplification of the targets in a pair of the optical channels.

19. The method of paragraph 18, wherein at least two of the reporters report amplification of the targets in a pair of the optical channels.

20. The method of paragraph 19, wherein each of the reporters reports amplification of the targets in a pair of the optical channels.

21. The method of paragraph 17, wherein at least one of the reporters reports amplification of the targets in only one of the optical channels.

22. The method of paragraph 17, wherein data representing amplification of the targets is collected in only two optical channels.

23. The method of paragraph 17, further comprising a step of identifying from the data a plurality of droplet populations each containing a different combination of the targets, wherein forming, amplifying, and collecting are performed such that each droplet population positive for two of the targets is at least substantially resolved from each droplet population positive for only one of the targets.

24. The method of any preceding paragraph, wherein the step of determining is based on at least R+2 identified droplet populations.

25. The method of paragraph 24, wherein the step of determining is based on at least 2R identified droplet populations.

26. The method of any preceding paragraph, wherein the droplets are all formed from the same bulk phase.

27. The method of any preceding paragraph, wherein the plurality of droplet populations include a plurality of droplet populations that are each positive for a different pair of the targets.

28. The method of any preceding paragraph, wherein the step of determining for at least one of the targets is based on a value that represents a sum of droplet counts from only one-half of the droplet populations.

29. The method of paragraph 28, wherein the step of determining for each target is based on a value that represents a sum of droplet counts from only one-half of the droplet populations.

The disclosure set forth above may encompass multiple distinct inventions with independent utility. Although each of these inventions has been disclosed in its preferred form(s), the specific embodiments thereof as disclosed and illustrated herein are not to be considered in a limiting sense, because numerous variations are possible. The subject matter of the inventions includes all novel and nonobvious combinations and subcombinations of the various elements, features, functions, and/or properties disclosed herein. The following claims particularly point out certain combinations and subcombinations regarded as novel and nonobvious. Inventions embodied in other combinations and subcombinations of features, functions, elements, and/or properties may be claimed in applications claiming priority from this or a related application. Such claims, whether directed to a different invention or to the same invention, and whether broader, narrower, equal, or different in scope to the original claims, also are regarded as included within the subject matter of the inventions of the present disclosure. Further, ordinal indicators, such as first, second, or third, for identified elements are used to distinguish between the elements, and do not indicate a particular position or order of such elements, unless otherwise specifically stated.

We claim:

**1**. A method of performing a multiplexed digital assay, the method comprising:

forming partitions that collectively contain R targets;

amplifying the R targets in the partitions;

collecting data representing amplification of each of the R targets in the partitions, all of the data being collected in fewer than R optical channels; and

determining a respective level of each of the R targets from the data, wherein each level is specific for a single target of the R targets, and wherein the level determined for at least one of the R targets is based in part on a partition count for a partition population positive for two of the R targets;

wherein the step of determining is based on at least R+2 identified partition populations.

**2**. The method of claim **1**, wherein R is at least three.

**3**. The method of claim **1**, wherein the data is collected in only two optical channels.

**4**. The method of claim **1**, wherein each optical channel represents a different waveband of emitted light.

**5**. The method of claim **1**, wherein each optical channel represents a different waveband of excitation light.

**6**. The method of claim **1**, wherein a different plurality of the partitions contains each different pair of the R targets.

**7**. The method of claim **1**, wherein at least 10% of the partitions contain a pair of the R targets.

**8**. The method of claim **1**, further comprising a step of identifying from the data a plurality of partition populations each positive for a different combination of the R targets.

**9**. The method of claim **1**, wherein the step of determining is based on at least 2R identified partition populations.

**10**. The method of claim **8**, wherein the step of identifying includes a step of resolving each partition population that is positive for exactly two of the R targets from each partition population that is positive for only one of the R targets.

**11**. A method of performing a multiplexed digital assay, the method comprising:

forming partitions that collectively contain R targets;

amplifying the R targets in the partitions;

collecting data representing amplification of each of the R targets in the partitions, all of the data being collected in fewer than R optical channels;

identifying from the data a plurality of partition populations each positive for a different combination of the R targets, wherein the step of identifying includes a step of resolving each partition population that is positive for exactly two of the R targets from each partition population that is positive for only one of the R targets; and

determining a respective level of each of the R targets from the data, wherein each level is specific for a single target of the R targets, and wherein the level determined for at least one of the R targets is based in part on a partition count for a partition population positive for two of the R targets.

**12**. The method of claim **11**, wherein R is at least three.

**13**. The method of claim **11**, wherein the data is collected in only two optical channels.

**14**. The method of claim **11**, wherein each optical channel represents a different waveband of emitted light.

**15**. The method of claim **11**, wherein a different plurality of the partitions contains each different pair of the R targets.

**16**. The method of claim **11**, wherein at least 10% of the partitions contain a pair of the R targets.

**17**. The method of claim **11**, wherein the step of determining is based on at least 2R identified partition populations.

**18**. A method of performing a multiplexed digital assay, the method comprising:

forming partitions that collectively contain R targets;

amplifying the R targets in the partitions;

collecting data representing amplification of each of the R targets in the partitions, all of the data being collected in fewer than R optical channels, wherein each optical channel represents a different waveband of excitation light; and

determining a respective level of each of the R targets from the data, wherein each level is specific for a single target of the R targets, and wherein the level determined for at least one of the R targets is based in part on a partition count for a partition population positive for two of the R targets.

**19**. A method of performing a multiplexed digital assay, the method comprising:

forming partitions that collectively contain R targets;

amplifying the R targets in the partitions;

collecting data representing amplification of each of the R targets in the partitions, all of the data being collected in fewer than R optical channels;

plotting the data such that partition populations containing different combinations of the R targets form a rectangular array, wherein the step of plotting is performed with respect to a pair of orthogonal axes, and wherein each partition population that is positive for only one of the R targets is arranged along a line that is at least generally parallel to one of the axes; and

determining a respective level of each of the R targets from the data, wherein each level is specific for a single target of the R targets, and wherein the level determined for at least one of the R targets is based in part on a partition count for a partition population positive for two of the R targets.

**20**. The method of claim **19**, wherein R is at least three.

**21**. The method of claim **19**, wherein the data is collected in only two optical channels.

**22**. The method of claim **19**, wherein each optical channel represents a different waveband of emitted light.

**23**. The method of claim **19**, wherein a different plurality of the partitions contains each different pair of the R targets.

**24**. The method of claim **19**, wherein at least 10% of the partitions contain a pair of the R targets.

**25**. The method of claim **19**, further comprising a step of identifying from the data a plurality of partition populations each positive for a different combination of the R targets.

**26**. The method of claim **25**, wherein the step of identifying includes a step of resolving each partition population that is positive for exactly two of the R targets from each partition population that is positive for only one of the R targets.

**27**. The method of claim **19**, wherein the step of determining is based on at least 2R identified partition populations.

**28**. The method of claim **19**, wherein the array is composed of a plurality of rows and columns, with each row and each column including at least two of the partition populations.

* * * * *

# EXHIBIT C

BIO-RAD

**Bio-Rad Laboratories, Inc.**

*Corporate Offices*
*1000 Alfred Nobel Drive*
*Hercules, California 94547*
*Phone: 510-724-7000*
*Fax: 510-741-4048*

September 6, 2023

Via UPS and Email
Padma Sundar
Chief Business Officer
ChromaCode, Inc.
2330 Faraday Avenue, Suite 100
Carlsbad, CA 92008
Email: [redacted]

Re: Notice of Potential Patent Infringement by ChromaCode's HDPCR Assays

Dear Ms. Sundar:

Bio-Rad is a global leader in developing innovative products for the life science research and clinical diagnostic markets and has a number of important patents. I am writing regarding the potential infringement of Bio-Rad-owned or licensed patents by ChromaCode.

Specifically, based on the public information currently available to and reviewed by Bio-Rad, ChromaCode's activities—including the making, using, offering to sell, and selling of ChromaCode's HDPCR Assays—appear to infringe the following patents.

| | |
|---|---|
| U.S. Patent No. 9,222,128 ('128 Patent) | "MULTIPLEXED DIGITAL ASSAYS WITH COMBINATORIAL USE OF SIGNALS" |
| U.S. Patent No. 9,921,154 ('154 Patent) | "MULTIPLEXED DIGITAL ASSAYS" |

U.S. Patent No. 9,222,128 describes, *inter alia*, "performing a multiplexed digital assay on a greater number of targets through combinatorial use of signals." ('128 Patent at Abstract.) The patent describes, *inter alia*, that "[e]ach signal may be a composite signal that represents two, three, four, or more reactions/assays and thus two, three, four, or more targets of the reactions/assays." (*Id.* at 6:43-45.)

U.S. Patent No. 9,921,154 describes, *inter alia*, "a system, including methods and apparatus, for performing a digital assay on a potentially greater number of targets through multiplexed detection of signals from reporters for two or more distinct targets in a common or shared channel ('the same optical channel')." ('154 Patent at 4:51-56.) The patent describes, *inter alia*, that: "For example, the intensity associated with one fluorophore, following reaction, may be lower or higher than the intensity(ies) associated with the other fluorophore(s). In some embodiments, one probe may be labeled with a different number of fluorophores than the other probe, and/or the probes may be located in slightly different local environments, creating a different level of fluorescence for each

probe following reaction. Alternatively, or in addition, both probes may be labeled with the same number of fluorophores (e.g., one fluorophore), but there may be more or less of one probe than the other in the sample, so that a greater or smaller signal is created when the reactions have occurred. In some cases, the fluorophores themselves might be different, with one more or less intrinsically fluorescent than the other (e.g., due to differences in extinction coefficient, quantum yield, etc.), so long as each fluorophore can be detected in the same channel." (*Id.* at 13:51-67.)

As non-limiting examples, Bio-Rad is providing exemplary infringement claim charts for the above-identified patents in Appendix 1-2 of this letter.

Additionally, Bio-Rad is providing you notice of the following patents and patent application:

| | |
|---|---|
| U.S. Patent No. 8,951,939 | "DIGITAL ASSAYS WITH MULTIPLEXED DETECTION OF TWO OR MORE TARGETS IN THE SAME OPTICAL CHANNEL" |
| U.S. Patent No. 9,745,617 | "DIGITAL ANALYTE ANALYSIS" |
| U.S. Patent Pub. No. 2022/0213530 | "DIGITAL ANALYTE ANALYSIS" |
| U.S. Patent No. RE41,780 | "CHEMICAL AMPLIFICATION BASED ON FLUID PARTITIONING IN AN IMMISCIBLE LIQUID" |

If you disagree with this notice of potential patent infringement, please provide the basis for that belief. Alternatively, if ChromaCode wishes to engage in potential licensing discussions with Bio-Rad, Bio-Rad is currently open to that discussion. Please contact me or Josh Shinoff, VP, Business Development ([redacted]) for more information.

This letter (including the attached appendices) is not intended to be an exhaustive or conclusive statement of Bio-Rad's positions. Bio-Rad's investigation is continuing. Bio-Rad may make different, additional, or alternative arguments. Nothing in this letter should be understood as construing any claims and/or broadening/narrowing/limiting any position Bio-Rad may take in the future or has taken in the past. Bio-Rad reserves all legal and equitable rights.

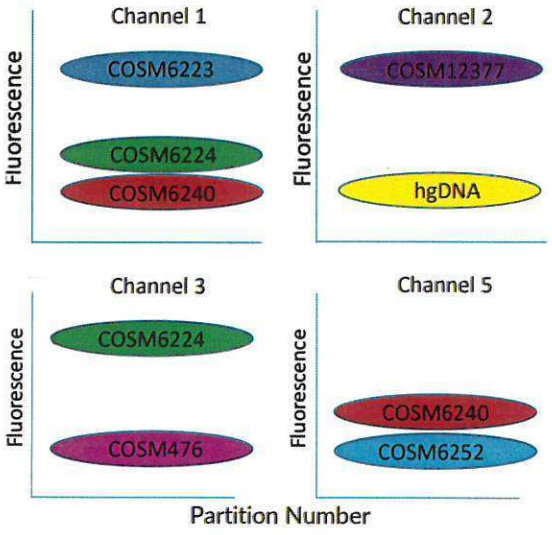
Sincerely,

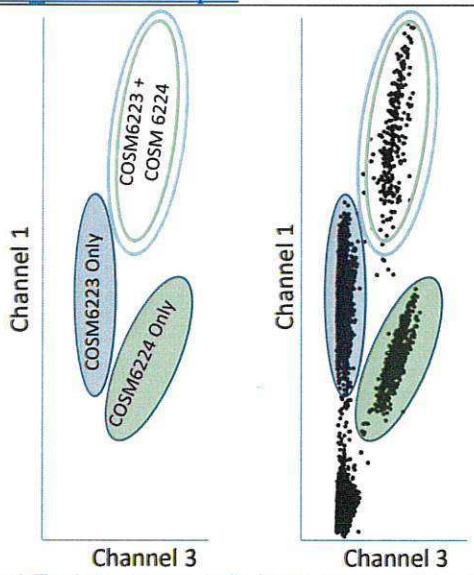
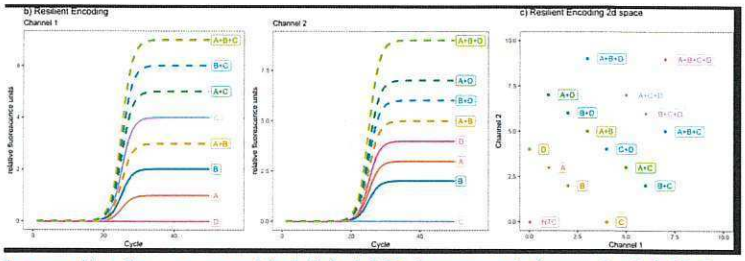
John J. Cassingham
VP, Asst. General Counsel
Bio-Rad Laboratories, Inc.
Email: [redacted]

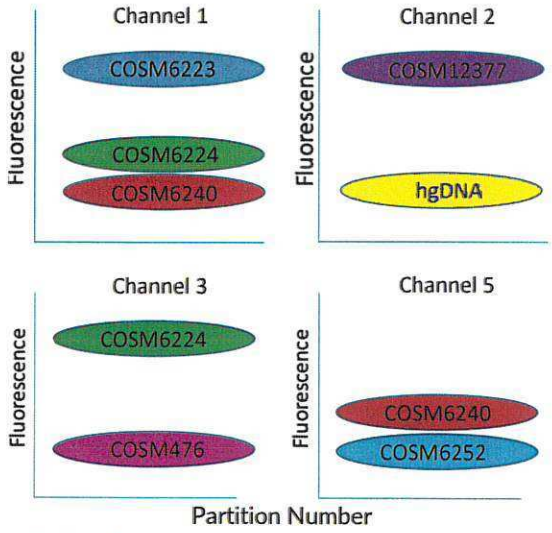
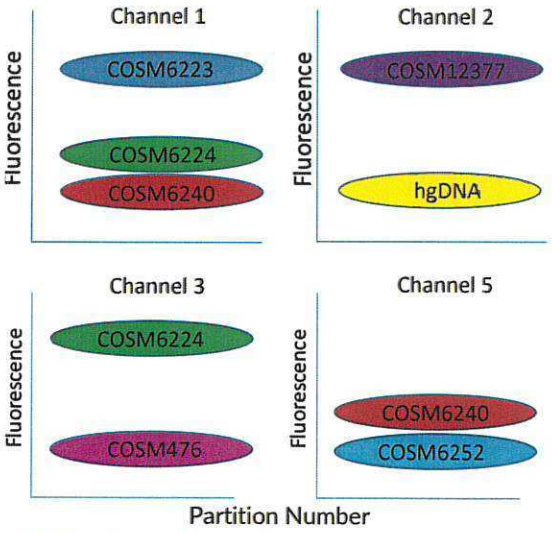
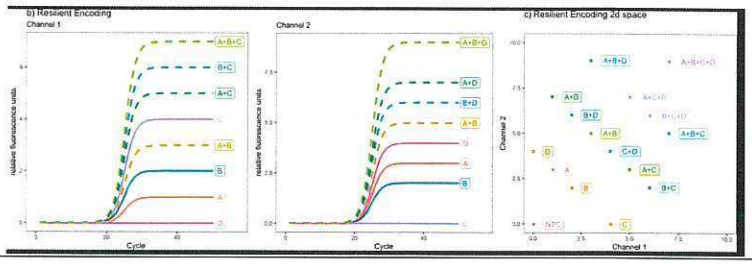
Cc: Josh Shinoff, VP, Business Development [via email: [redacted]]

# Appendix 1

**U.S. Patent No. 9,222,128**

| U.S. Patent No. 9,222,128, Claim 1 | ChromaCode's HDPCR |
|---|---|
| 1. A method of performing a multiplexed digital amplification assay, the method comprising: | ChromaCode has developed a research use only (RUO) high-definition PCR (HDPCR™) assay on a digital PCR instrument for multiplexed detection of 14 DNA variants and 15 RNA fusion variants relevant in non-small cell lung cancer (NSCLC) samples. The assay is https://www.chromacode.com/wp-content/uploads/2022/04/635477.1_-AACR-2022-poster_21mar2022.pdf |
| amplifying more than R targets in partitions; |  <br>**Figure 1. Resilient encoding strategy for molecular variants: The above four schematic plots show how multiple targets are encoded in a single channel using the HDPCR™ technology.**<br>ChromaCode's HDPCR[1,2] technology enables different variants to generate a signal at different intensity levels in single color channel, allowing for greater than N targets in N color channels. In contrast, resilient coding generates a signal in more than one color channel to create a form of error-detecting code.<br>https://www.chromacode.com/wp-content/uploads/2022/04/635477.1_-AACR-2022-poster_21mar2022.pdf |
| creating R signals representative of light detected in R different wavelength regimes from the partitions, where R≧2; and | See previous row. |

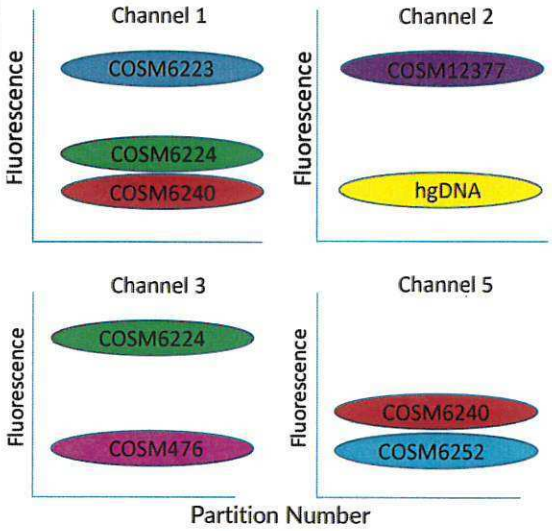
| | |
|---|---|
| calculating an average level of each target in the partitions based on the R signals, |  <br>Table 2: Combined results of both contrived-cfDNA and contrived-FFPE samples with breakdown by representative DNA and RNA variants.<br>https://www.chromacode.com/wp-content/uploads/2022/04/635477.1_-AACR-2022-poster_21mar2022.pdf |
| wherein the level calculated accounts for a coincidence of all possible combinations of the more than R targets in the same individual partitions; |  <br>Figure 2. The above schematic plot (left) and the experimental data (right, 20% MAF, contrived with cfDNA background) show both a resilient encoding strategy and multiple levels of signals in HDPCR. COSM6224 is resiliently encoded in both channels 1 and 3. When both variants co-occur HDPCR is able resolve partitions that contain both and those that contain only one.<br>https://www.chromacode.com/wp-content/uploads/2022/04/635477.1_-AACR-2022-poster_21mar2022.pdf<br>  <br>https://pubs.acs.org/doi/10.1021/acs.analchem.0c04626 |

| | |
|---|---|
| wherein the more than R targets include three targets, and |  <br>**Figure 1. Resilient encoding strategy for molecular variants: The above four schematic plots show how multiple targets are encoded in a single channel using the HDPCR™ technology.**<br>https://www.chromacode.com/wp-content/uploads/2022/04/635477.1_-AACR-2022-poster_21mar2022.pdf |
| wherein the average levels of the three targets are calculated based on light detected from only two fluorophores associated with probes that bind to amplicons of the three targets during amplification. |  <br>**Figure 1. Resilient encoding strategy for molecular variants: The above four schematic plots show how multiple targets are encoded in a single channel using the HDPCR™ technology.**<br>https://www.chromacode.com/wp-content/uploads/2022/04/635477.1_-AACR-2022-poster_21mar2022.pdf<br>  |

| | |
|---|---|
| | https://pubs.acs.org/doi/10.1021/acs.analchem.0c04626 |

# Appendix 2

**U.S. Patent No. 9,921,154**

| U.S. Patent No. 9,921,154, Claim 1 | Chromacode's HDPCR |
|---|---|
| 1. A method of performing a multiplexed digital assay, the method comprising: | ChromaCode has developed a research use only (RUO) high-definition PCR (HDPCR™) assay on a digital PCR instrument for multiplexed detection of 14 DNA variants and 15 RNA fusion variants relevant in non-small cell lung cancer (NSCLC) samples. The assay is https://www.chromacode.com/wp-content/uploads/2022/04/635477.1_-AACR-2022-poster_21mar2022.pdf |
| forming partitions that collectively contain R targets; |  <br>Figure 1. Resilient encoding strategy for molecular variants: The above four schematic plots show how multiple targets are encoded in a single channel using the HDPCR™ technology.<br>https://www.chromacode.com/wp-content/uploads/2022/04/635477.1_-AACR-2022-poster_21mar2022.pdf |
| amplifying the R targets in the partitions; | Samples were run with the HDPCR NSCLC RUO assay on the Applied Biosystems QuantStudio™ Absolute Q Digital PCR system, and data analysis was performed with proprietary analysis algorithms.<br>https://www.chromacode.com/wp-content/uploads/2022/04/635477.1_-AACR-2022-poster_21mar2022.pdf |

collecting data representing amplification of each of the R targets in the partitions,

| EGFR L858R | | Detected | |
|---|---|---|---|
| COSM6224 | | + | - |
| Spiked-in | + | 167 | 0 |
| | - | 0 | 211 |

| EML4-ALK variant 1 | | Detected | |
|---|---|---|---|
| COSMAB274722.1 | | + | - |
| Spiked-in | + | 83 | 0 |
| | - | 3 | 406 |

| KRAS G12C | | Detected | |
|---|---|---|---|
| COSM516 | | + | - |
| Spiked-in | + | 37 | 0 |
| | - | 0 | 70 |

| MET exon 14 skipping | | Detected | |
|---|---|---|---|
| | | + | - |
| Spiked-in | + | 86 | 1 |
| | - | 5 | 403 |

| EGFR T790M | | Detected | |
|---|---|---|---|
| COSM6240 | | + | - |
| Spiked-in | + | 122 | 1 |
| | - | 0 | 254 |

| KIF5B-RET (K15:R12) | | Detected | |
|---|---|---|---|
| COSMAB795250.1 | | + | - |
| Spiked-in | + | 86 | 0 |
| | - | 2 | 409 |

| EGFR Exon20 H773dup | | Detected | |
|---|---|---|---|
| COSM12377 | | + | - |
| Spiked-in | + | 42 | 0 |
| | - | 0 | 326 |

| MPRIP-NTRK1 (M21:N14) | | Detected | |
|---|---|---|---|
| COSMKF724384.1 | | + | - |
| Spiked-in | + | 87 | 0 |
| | - | 5 | 401 |

| EGFR E746_A750del | | Detected | |
|---|---|---|---|
| COSM4223 | | + | - |
| Spiked-in | + | 126 | 0 |
| | - | 0 | 252 |

| EZR-ROS1 (E10-R34) | | Detected | |
|---|---|---|---|
| COSMAB795246.1 | | + | - |
| Spiked-in | + | 81 | 1 |
| | - | 0 | 408 |

Table 2: Combined results of both contrived-cfDNA and contrived-FFPE samples with breakdown by representative DNA and RNA variants.

https://www.chromacode.com/wp-content/uploads/2022/04/635477.1_-AACR-2022-poster_21mar2022.pdf

all of the data being collected in fewer than R optical channels; and

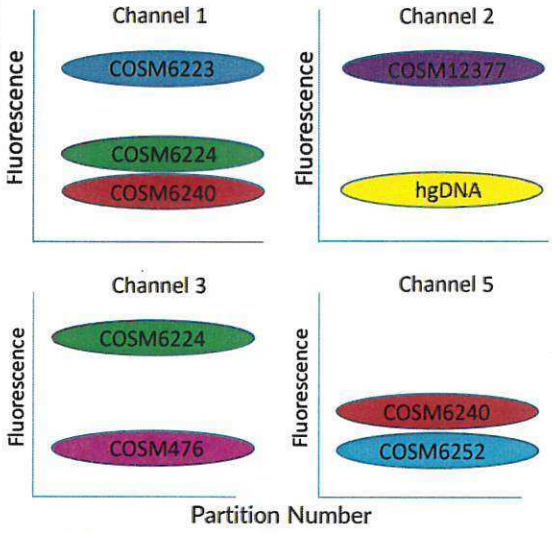
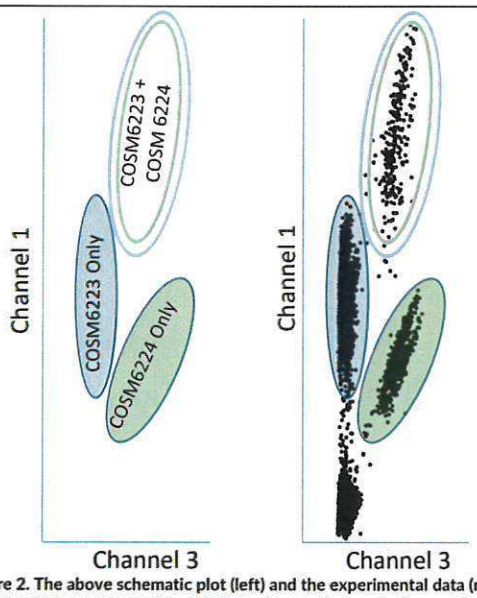



**Figure 1. Resilient encoding strategy for molecular variants: The above four schematic plots show how multiple targets are encoded in a single channel using the HDPCR™ technology.**

ChromaCode's HDPCR[1,2] technology enables different variants to generate a signal at different intensity levels in single color channel, allowing for greater than N targets in N color channels. In contrast, resilient coding generates a signal in more than one color channel to create a form of error-detecting code.

https://www.chromacode.com/wp-content/uploads/2022/04/635477.1_-AACR-2022-poster_21mar2022.pdf

| | |
|---|---|
| determining a respective level of each of the R targets from the data, |  <br>EGFR L858R COSM6224: Spiked-in + 167 0; Spiked-in - 0 211<br>EML4-ALK variant 1 COSMAB274722.1: Spiked-in + 83 0; Spiked-in - 3 406<br>KRAS G12C COSM516: Spiked-in + 37 0; Spiked-in - 0 70<br>MET exon 14 skipping: Spiked-in + 86 1; Spiked-in - 5 403<br>EGFR T790M COSM6240: Spiked-in + 122 1; Spiked-in - 0 254<br>KIF5B-RET (K15:R12) COSMAB795250.1: Spiked-in + 86 0; Spiked-in - 2 409<br>EGFR Exon20 H773dup COSM12377: Spiked-in + 42 0; Spiked-in - 0 326<br>MPRIP-NTRK1 (M21:N14) COSMKF724384.1: Spiked-in + 87 0; Spiked-in - 5 401<br>EGFR E746_A750del COSM6223: Spiked-in + 126 0; Spiked-in - 0 252<br>EZR-ROS1 (E10-R34) COSMAB795246.1: Spiked-in + 81 1; Spiked-in - 0 408<br>Table 2: Combined results of both contrived-cfDNA and contrived-FFPE samples with breakdown by representative DNA and RNA variants.<br>https://www.chromacode.com/wp-content/uploads/2022/04/635477.1_-AACR-2022-poster_21mar2022.pdf |
| wherein each level is specific for a single target of the R targets, |  <br>EGFR L858R COSM6224: Spiked-in + 167 0; Spiked-in - 0 211<br>EML4-ALK variant 1 COSMAB274722.1: Spiked-in + 83 0; Spiked-in - 3 406<br>KRAS G12C COSM516: Spiked-in + 37 0; Spiked-in - 0 70<br>MET exon 14 skipping: Spiked-in + 86 1; Spiked-in - 5 403<br>EGFR T790M COSM6240: Spiked-in + 122 1; Spiked-in - 0 254<br>KIF5B-RET (K15:R12) COSMAB795250.1: Spiked-in + 86 0; Spiked-in - 2 409<br>EGFR Exon20 H773dup COSM12377: Spiked-in + 42 0; Spiked-in - 0 326<br>MPRIP-NTRK1 (M21:N14) COSMKF724384.1: Spiked-in + 87 0; Spiked-in - 5 401<br>EGFR E746_A750del COSM6223: Spiked-in + 126 0; Spiked-in - 0 252<br>EZR-ROS1 (E10-R34) COSMAB795246.1: Spiked-in + 81 1; Spiked-in - 0 408<br>Table 2: Combined results of both contrived-cfDNA and contrived-FFPE samples with breakdown by representative DNA and RNA variants.<br>https://www.chromacode.com/wp-content/uploads/2022/04/635477.1_-AACR-2022-poster_21mar2022.pdf |
| and wherein the level determined for at least one of the R targets is based in part on a partition count for a partition population positive for two of the R targets; |  <br>COSM6223 + COSM 6224<br>COSM6223 Only<br>COSM6224 Only<br>Channel 1<br>Channel 3<br>Channel 1<br>Channel 3<br>**Figure 2. The above schematic plot (left) and the experimental data (right, 20% MAF, contrived with cfDNA background) show both a resilient encoding strategy and multiple levels of signals in HDPCR. COSM6224 is resiliently encoded in both channels 1 and 3. When both variants co-occur HDPCR is able resolve partitions that contain both and those that contain only one.** |

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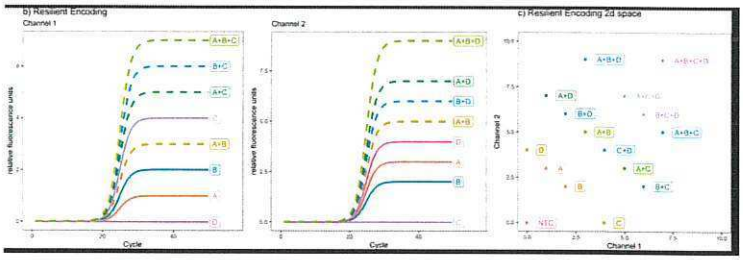
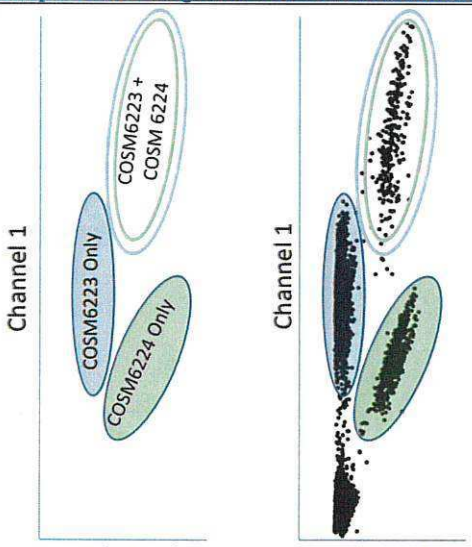
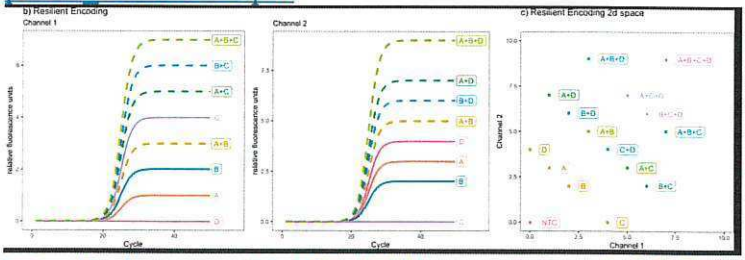
|---|---|
| | https://www.chromacode.com/wp-content/uploads/2022/04/635477.1_-AACR-2022-poster_21mar2022.pdf<br>  <br>https://pubs.acs.org/doi/10.1021/acs.analchem.0c04626 |
| wherein the step of determining is based on at least R+2 identified partition populations. |  <br><br>**Figure 2. The above schematic plot (left) and the experimental data (right, 20% MAF, contrived with cfDNA background) show both a resilient encoding strategy and multiple levels of signals in HDPCR. COSM6224 is resiliently encoded in both channels 1 and 3. When both variants co-occur HDPCR is able resolve partitions that contain both and those that contain only one.**<br>https://www.chromacode.com/wp-content/uploads/2022/04/635477.1_-AACR-2022-poster_21mar2022.pdf<br>  <br>https://pubs.acs.org/doi/10.1021/acs.analchem.0c04626 |