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Provisional Application for Patent Cover Sheet

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c)

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Title of Invention	DIGITAL ASSAYS WITH MULTIPLEXED DETECTION OF TWO OR MORE TARGETS IN THE SAME CHANNEL
Attorney Docket Number (if applicable)	QLI 325P

Correspondence Address

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

◉ No.

◯ Yes, the name of the U.S. Government agency and the Government contract number are:

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Entity Status

Applicant claims small entity status under 37 CFR 1.27

- (●) Yes, applicant qualifies for small entity status under 37 CFR 1.27
- ( ) No

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Signature	/james r abney/			Date (YYYY-MM-DD)	2011-07-12
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DIGITAL ASSAYS WITH MULTIPLEXED DETECTION OF TWO OR MORE TARGETS IN THE SAME CHANNEL

Cross-References

5 This application incorporates by reference in their entireties for all purposes the following materials: U.S. Patent No. 7,041,481, issued May 9, 2006; U.S. Patent Application Publication No. 2010/0173394 A1, published July 8, 2010; and Joseph R. Lakowicz, PRINCIPLES OF FLUORESCENCE SPECTROSCOPY (2nd Ed. 1999).

Introduction

10 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,
15 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
20 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

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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
5 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
10 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
15 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"). If a detector for a digital PCR assay can distinguishably measure the fluorescence emitted by N different dyes, then the assay is
20 effectively capable of measuring N 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 and apparatus, for performing a digital assay with multiplexed detection of two or more distinct targets in the same channel.

Brief Description of the Drawings

Figure 1 is a flowchart of an exemplary method of performing a digital assay with multiplexed detection of two or more distinct targets in the same channel, in accordance with aspects of the present disclosure.

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

Figure 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 digital PCR assay, in accordance with aspects of the present disclosure.

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

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Figure 5 is an exemplary graph showing droplet (or peak) intensities for a C61/RPP30 droplet assay, where data from reporters for C61 and RPP30 are collected together in the same channel and plotted as a function of intensity in a FAM channel (vertical axis) versus intensity in a VIC channel (horizontal axis).

5 Figure 6 is a graph showing FAM intensities for the C61/RPP30 droplet assay of Figure 5 for twelve different sets of assay conditions (denoted, from left to right, 55.0, 55.2, …, 64.6, and 64.9).

Figure 7 is an exemplary graph showing droplet (or peak) intensities for a C63/RPP30 droplet assay, where data from reporters for C63 and RPP30 are collected
10 together in the same channel and plotted as a function of intensity in a FAM channel (vertical axis) versus intensity in a VIC channel (horizontal axis).

Figure 8 shows FAM intensities (top panel) and VIC intensities (bottom panel) for the C63/RPP30 droplet assay of Figure 7 for twelve different sets of assay conditions (denoted, from left to right, 55.0, 55.2, …, 64.6, and 64.9).

15

Detailed Description

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 detection channel ("the same channel"). The reporters may include the same
20 fluorophore, such as FAM or VIC, or different fluorophores with similar spectral characteristics, so that light from each reporter may be collected simultaneously in the same 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 (assay1) target, those with a second assay (assay2) 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 5 estimated by taking into account the total number of droplets in each population. Concentrations 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) and copy 10 number variation (CNV), among other quantities. Copy number variation is a structural variation in the genome, such as deletions, duplications, translocations, and/or inversions, that 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 15 may involve analysis of more than two targets in the same channel. For example, an assay for three targets would 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 other targets may be analyzed in other channels (e.g., a VIC channel). Three targets, two in a first channel and one in a second channel, 20 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.

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

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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.

Figure 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.

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, 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 reporters for

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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 probe that includes a nucleic acid (e.g., an oligonucleotide) and 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.

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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, 5 or the like.

Sample reactions. 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 10 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 15 reaction and/or ligase chain reaction. Accordingly, a plurality of amplification reactions for a plurality 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. 20 In some examples, the conditions may include thermally cycling the partitions to promote a polymerase chain reaction and/or ligase chain reaction.

Signal creation. Signals may be created for each of the partitions that are representative of light detected from the partitions, indicated at 48. The signals may

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represent aspects of light, such as the intensity of the light, detected in the same channel (i.e., in the same wavelength range (color regime) such as for FAM or VIC) from reporters for two or more distinct targets. The signals optionally may include data collected in one or more different channels (i.e., in different wavelength ranges (color
5 regimes) from reporters for the same and/or different targets). The light detected from each reporter may be light emitted from a fluorophore. The light detected in a given channel may be detected with the same sensor at the same time such that light detected from different reporters is summed or accumulated without attribution to a particular reporter. Thus, the signal may be a composite signal that represents two,
10 three, four, or more reactions and thus two, three, four, or more targets of the reactions.

The signal 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 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
15 corresponding to the two or more particular reactions is present in the partition. The strength of the signal corresponding to the reporters 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 strength may vary among the partitions according to whether at least one of the particular reactions occurred or did
20 not occur (e.g., above a threshold extent) and at least one of the particular targets is present or absent in each partition.

Partitions may be analyzed and signals created at any suitable time(s). Exemplary times include at the end of an assay (endpoint assay), when reactions have

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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.

Number of positives. 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 5 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 10 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 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, assuming that each population is distinguishable. 15 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

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	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

Concentration estimate. A concentration of each target may be estimated, indicated at 52. The concentration of each target may be estimated based on the respective numbers of partitions positive for the target alone and for the target in
5 combination with any other target(s). The calculation may be based on each target having a Poisson distribution among the droplets. The concentrations may, for example, be estimated by finding solutions to a series of linear equations. The total number of partitions may be counted or, in some cases, estimated. The droplet data further may be used (e.g., directly and/or as concentration data) to estimate copy number (CN) and
10 copy number variation (CNV), using any suitable algorithms such as those described elsewhere in the present disclosure.

Figure 2 shows an exemplary apparatus 60 for performing the digital assay of Figure 1. Apparatus 60 may include a partitioning assembly, such as a droplet generator 62 (“DG”), a thermal incubation assembly, such as a thermocycler 64 (“TC”),
15 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 movement or transfer of material, such as fluid (e.g., a

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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 is
5 transferred manually.

Apparatus 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
10 processor 68 to estimate concentrations of the targets.

II. Examples

This section presents selected aspects and embodiments of the present disclosure related to methods of performing digital assays with multiplexed detection of two or more targets in the same channel.

15 *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.

20 Figure 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

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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 5 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.

10 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) 15 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 20 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

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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.

Figure 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 Figure 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

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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):

5
- 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.

10
- 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.

15
- 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.

20
- 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.

25 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.

30 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.

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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
5 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.

<u>Example 2.</u> *Digital PCR Assay to Assess Copy Number of C61*

This example describes a first exemplary digital PCR assay, in which
10 multiplexing in a single channel is used to assess copy number of the C61 gene; see Figures 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.

15 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
20 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.

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Figure 5 is a graph showing data for the exemplary C61/RPP30 system. Specifically, Figure 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. 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.,
5 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
10 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).

Figure 6 shows FAM intensities for the C61/RPP30 system of Figure 5 for twelve different sets of experimental conditions (denoted, from left to right, 55.0, 55.2, …, 64.6,
15 and 64.9). 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 Figure 5 correspond to one of the conditions in this plot.

<u>Example 3.</u> Digital PCR Assay to Assess Copy Number of C63

This example describes a second exemplary digital PCR assay, in which
20 multiplexing in a single channel is used to assess copy number of the C63 gene; see Figures 7 and 8. Specifically, signals from two probes, one for the gene of interest, C63,

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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.

Figure 7 is a graph showing data for the exemplary C63/RPP30 system. Like Figure 5, Figure 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 Figure 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).

Figure 8 shows FAM intensities (top panel) and VIC intensities (bottom panel) for the C63/RPP30 system of Figure 7 for twelve different sets of experimental conditions. 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 Figure 7 correspond to one of the conditions in these plots.

Example 4. *Selected Embodiments*

This example describes selected aspects and embodiments related to digital assays with multiplexed detection of two or more targets in the same channel, in accordance with aspects of the present disclosure, presented without limitation as a series of numbered paragraphs.

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1. A method of performing a digital assay, comprising (A) separating a sample containing at least two types of targets into partitions containing a reporter for each type of target; (B) detecting from each partition a signal representing combined emission of light from the reporters for each type of target, wherein each reporter
5 provides a portion of the signal having an intensity that varies according to whether or not the corresponding type of target is present in a partition; (C) counting a number of the partitions that are positive or that are negative for each type of target based on the signal; and (D) determining a quantity representative of a level of at least one type of target in the sample.

10 2. The method of paragraph 1, wherein the quantity representative of a level is a concentration of the target in the sample.

3. The method of paragraph 1, wherein the step of determining a quantity representative of a level further comprises (D1) ascertaining a total number of partitions based on the signal; and (D2) obtaining a concentration of the target based on the
15 counted number of partitions and the total number of partitions.

4. The method of paragraph 1, wherein the quantity representative of a level is a copy number of the target in the sample.

5. The method of paragraph 1, wherein the step of determining a quantity representative of a level further comprises obtaining a copy number of a first target by
20 comparing the number of partitions positive for the first target with the number of partitions positive for a second target, the first and second targets being among the at least two types of targets contained in the sample.

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6. The method of paragraph 5, wherein the copy number of the second target is known, and wherein the copy number of the first target is determined as an absolute number (e.g., one, two, three, etc.) by calculating the ratio of the number of partitions positive for the first target to the number of partitions positive for the second target and multiplying the ratio by the copy number of the second target.

7. The method of paragraph 5, wherein the copy number of the second target is unknown, and wherein the copy number of the first target is obtained relative to the copy number of the second target as the ratio of the number of partitions positive for the first target to the number of partitions positive for the second target.

8. The method of paragraph 1, wherein the reporters each are labeled with the same fluorophore.

9. The method of paragraph 8, wherein the fluorophore is selected from the group consisting of FAM, VIC, ROX, TAMRA, and JOE.

10. The method of paragraph 1, the signal being a first signal, further comprising (E) detecting from each partition a second signal representing emission of light from an additional reporter for an additional type of target, the additional reporter providing at least a portion of a second signal having an intensity that varies according to whether or not the corresponding additional type of target is present in a partition, wherein the first signal and second signal correspond to emission of light in different wavelength regimes; (F) counting a number of the partitions that are positive or that are negative for the additional type of target based on the second signal; and (G) determining a quantity representative of a level of the additional type of target in the sample.

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11. The method of paragraph 10, wherein the first signal is collected from a first type of fluorophore and the second signal is collected from a second type of fluorophore.

12. The method of paragraph 11, wherein the first type of fluorophore is one of FAM, VIC, ROX, TAMRA, and JOE, and wherein the second type of fluorophore is another of FAM, VIC, ROX, TAMRA, and JOE.

13. The method of paragraph 1, wherein the partitions include a first probe reporting a presence or absence of a first target in individual partitions, a second probe reporting a presence or absence of a second target in individual partitions, and a third probe reporting a presence or absence of a third target in individual partitions, and wherein a signal from the first and second probes represents light emitted in a first wavelength regime and a signal from the third probe represents light emitted in a second wavelength regime distinct from and nonoverlapping with the first wavelength regime.

14. The method of paragraph 1, 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 types of targets.

15. The method of paragraph 1, wherein the step of separating a sample forms one or more partitions containing no copies of a target for each of the types of targets.

16. The method of paragraph 1, wherein the partitions are droplets.

17. The method of paragraph 1, wherein the partitions have a substantially same volume relative to one another.

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18. The method of paragraph 1, further comprising a step of performing an amplification reaction in one or more of the partitions before the step of detecting a signal.

19. The method of paragraph 18, wherein the step of performing an amplification reaction includes a step of thermally cycling partitions to perform a polymerase chain reaction.

20. The method of paragraph 1, wherein each target is a nucleic acid.

21. The method of paragraph 1, wherein the signal is representative of light detected from fluid carrying droplets through an examination region.

22. An apparatus for performing the method of any of paragraphs 1-21 on a sample containing at least two targets, comprising (A) a partitioning assembly; (B) a thermal incubation assembly; (C) a detection assembly; and (D) a data processing assembly configured (i) to communicate with and control operation of at least one of the partitioning assembly, the thermal incubation assembly, and the detection assembly, and (ii) to determine a quantity representative of as level of at least one type of target in the sample.

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.

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Abstract

System, including methods and apparatus, for performing a digital assay with multiplexed detection of two or more distinct targets in the same channel.

Fig. 1

40

42 PREPARE SAMPLE

44 SEPARATE SAMPLE INTO PARTITIONS

46 PERFORM REACTION

48 CREATE SIGNAL IN A SINGLE CHANNEL REPRESENTATIVE OF LIGHT DETECTED FROM PARTITIONS

50 DETERMINE FROM SIGNAL A NUMBER OF PARTITIONS POSITIVE FOR EACH OF TWO OR MORE TARGETS

52 ESTIMATE CONCENTRATION OF EACH TARGET

Fig. 2

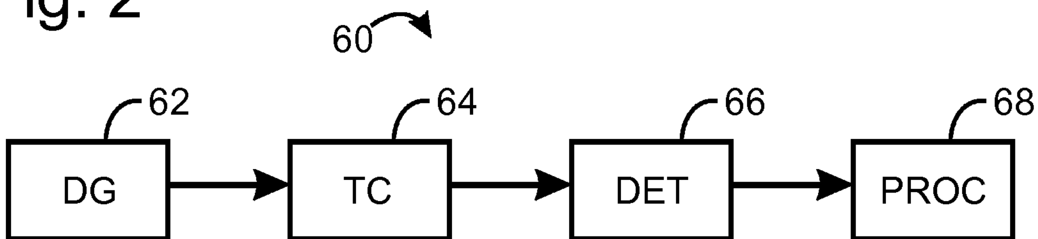

Fig. 3

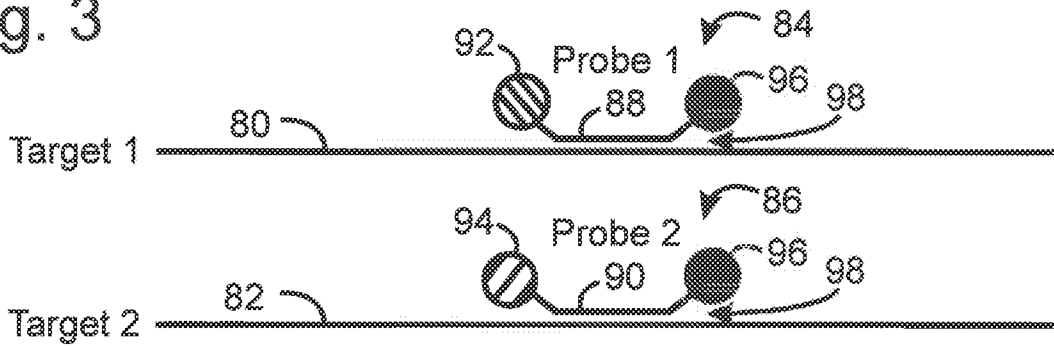

Fig. 4

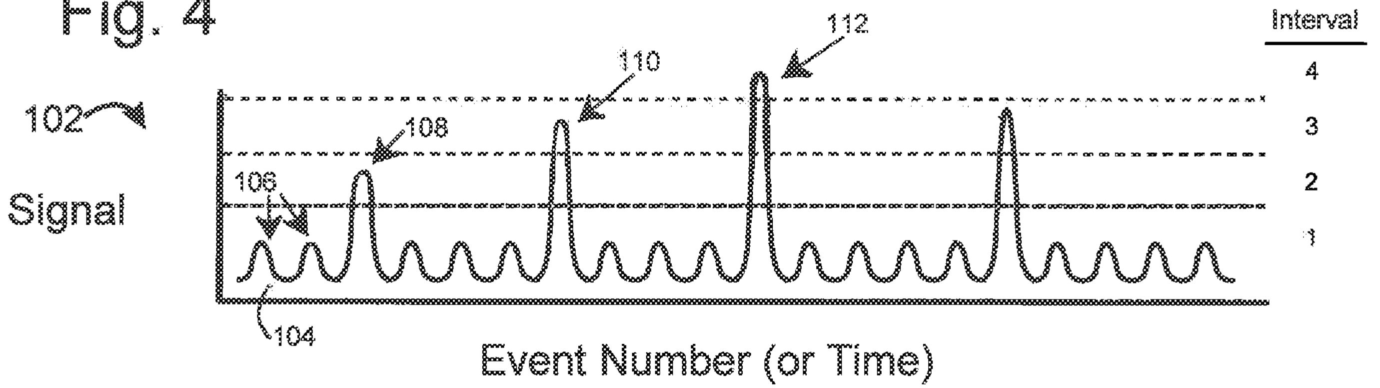

Fig. 5

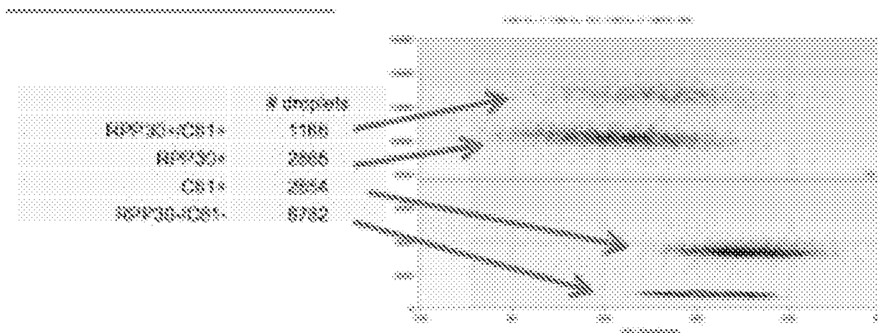

Fig. 6

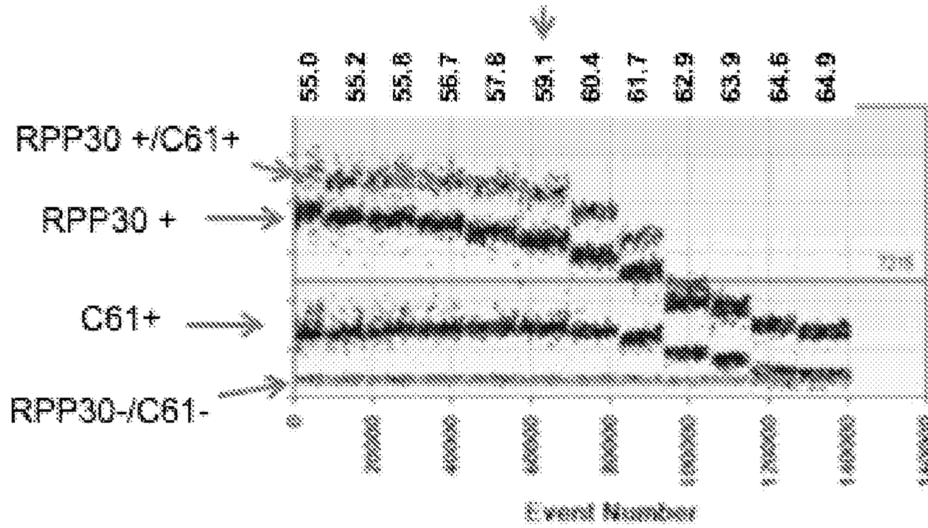

Fig. 7

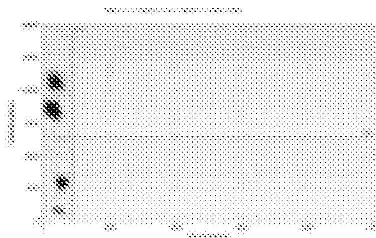

Fig. 8