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FILING RECEIPT



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**** SMALL ENTITY ****

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DIGITAL ASSAYS WITH MULTIPLEXED DETECTION OF TWO OR MORE TARGETS IN THE SAME CHANNEL

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Provisional Application for Patent Cover Sheet

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All Inventors Must Be Listed – Additional Inventor Information blocks may be generated within this form by selecting the **Add** button.**Add****Title of Invention**

DIGITAL ASSAYS WITH MULTIPLEXED DETECTION OF TWO OR MORE TARGETS IN THE SAME CHANNEL

Attorney Docket Number (if applicable)

QLI 325P1

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Signature	/ james r abney /			Date (YYYY-MM-DD)	2011-07-20
First Name	JAMES R.	Last Name	ABNEY	Registration Number (If appropriate)	42253

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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; U.S. Provisional Patent Application Serial No. 61/507,082, filed July 12, 2011; 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, 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.

15

20 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 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 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, 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 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.

5

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

10

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.

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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.

20

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.

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 (anneal temperatures, denoted, from left to right, 55.0, 55.2, ..., 64.6, and 64.9 degrees Celsius).

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 (anneal temperatures, denoted, from left to right, 55.0, 55.2, ..., 64.6, and 64.9 degrees
15 Celsius).

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
20 shared detection channel ("the same channel"). The reporters may include the same 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). In some cases, preliminary assays may be run, using aliquots of the actual sample and/or materials representative of the sample, to determine suitable or optimal assay conditions, such as anneal temperatures capable of allowing reactions to run substantially toward or to completion in thermocycling assays. 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 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 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 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, 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:

- 5 (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.

Figure 1 shows a flowchart of an exemplary method 40 of performing a digital
10 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
15 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
20 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 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.

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.

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 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 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.

Signal creation. Signals may be created for each of the partitions that are
5 representative of light detected from the partitions, indicated at 48. The signals may 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
10 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,
15 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
20 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 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).

- 5 Exemplary 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.

- 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
- 10 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
- 15 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.
- 20 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

Concentration estimate. A concentration of each target may be estimated,

5 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 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
10 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 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

15 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”), 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
5 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
10 unconnected to the other assemblies, such that, for example, material/data is 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
15 detected from the droplets with detector 66. The signals may be processed by 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
20 two or more targets in the same channel.

Example 1. Exemplary Assay Formats

The example describes exemplary formats and conditions for performing digital assays with multiplexed detection.

The assays presented herein may be performed using any suitable techniques. Exemplary techniques, including assay formats and conditions, are described without limitation in Appendix A and U.S. Patent Application Serial No. 12/976,827, filed December 22, 2010, among others. These techniques may be performed using any
5 suitable apparatus, including apparatus described without limitation in U.S. Patent No. 7,041,481, issued May 9, 2006; and U.S. Patent Application Publication No. 2010/0173394 A1, published July 8, 2010. All of the patent documents cross-referenced in this section are hereby incorporated by reference in their entireties.

Example 2. Digital PCR Assays with Multiplexed Detection in the Same Channel

10 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.

Figure 3 shows a pair of targets 80, 82 ("Target 1" and "Target 2") and
15 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
20 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.

5 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)
10 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
15 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.
20 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
5 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
10 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
15 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 and 2 are present in each droplet. In particular, the strength or intensity of the signal in a
20 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 3. *Digital PCR Assay to Assess Copy Number of C61*

5 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 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
10 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.

15 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.

20 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:

- 5 • 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).
- 10 • 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).
- 15 • 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).
- 20 • 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 2, 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).

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, and 64.9). These different conditions may correspond to anneal temperatures, in degrees Celsius, preceding the extend step(s) in a thermocycling assay. 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. This variety of conditions may correspond to relatively lower anneal temperatures (e.g., below about 60 degrees Celsius), in a thermocycling assay, sufficient to allow enough binding between template and primers for the thermocycling assay to run substantially toward or to completion. The data shown in Figure 5 correspond to one of the conditions in this plot. Less desirable conditions, such as relatively higher anneal temperatures (e.g., above about 60 degrees Celsius), may not allow sufficient resolution between populations, for example, because the signal from different partitions smears out as the extent of reaction varies from partition to partition.

Example 4. *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 Figures 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.

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 5. *Exemplary Assay Results*

The example describes exemplary assay results, for a variety of targets, under a variety of conditions. The results are described without limitation in Appendix B. Some of the results correspond to and/or elaborate upon results presented in Examples 3 and 4.

Example 6. *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.

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 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.

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 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 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 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.

10 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.

15 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.

20 8. The method of paragraph 1, wherein the step of counting a number of the partitions that are positive or that are negative for each type of target includes treating a partition as positive if the partition produces a signal that is above a preselected (e.g., threshold) level and treating a partition as negative if the partition produces a signal that is below the preselected level.

9. The method of paragraph 8, wherein the signal is a fluorescence signal and the preselected level is a preselected fluorescence intensity.

10. The method of paragraph 1, wherein the step of counting a number of the partitions that are positive or that are negative for each type of target includes using an
5 expectation maximization algorithm to estimate the numbers of partitions that are positive and/or negative for at least one of the targets.

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

12. The method of paragraph 11, wherein the fluorophore is selected from the
10 group consisting of FAM, VIC, ROX, TAMRA, and JOE.

13. 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
15 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
20 sample.

14. The method of paragraph 13, wherein the first signal is collected from a first type of fluorophore and the second signal is collected from a second type of fluorophore.

15. The method of paragraph 14, 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.

16. The method of paragraph 1, wherein the partitions include a first probe
5 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
10 second wavelength regime distinct from and nonoverlapping with the first wavelength regime.

17. 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.

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

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

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

21. 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.

22. The method of paragraph 21, wherein the step of performing an amplification reaction includes a step of thermally cycling partitions to perform a polymerase chain reaction.

23. The method of paragraph 22, wherein the amplification reaction includes
5 sequential melt, anneal, and extend steps, and wherein the anneal steps are performed at a sufficiently low temperature to allow sufficient annealing to occur so that the reaction can run to completion during subsequent extend step(s).

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

25. The method of paragraph 24, further comprising a step of cleaving the
10 targets into fragments prior to, or concomitant with, the step of separating a sample into partitions, so that the partitions are enriched in fragments of the targets rather than uncleaved (full-size) targets.

26. The method of paragraph 25, wherein the step of cleaving the targets includes a step of exposing the targets to one or more restriction enzymes.

15 27. The method of paragraph 26, wherein the assay is for one or more genes of interest, and wherein the restriction enzymes are selected so as not to cleave the targets within the genes of interest.

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

20 29. An apparatus for performing the method of any of paragraphs 1-28 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

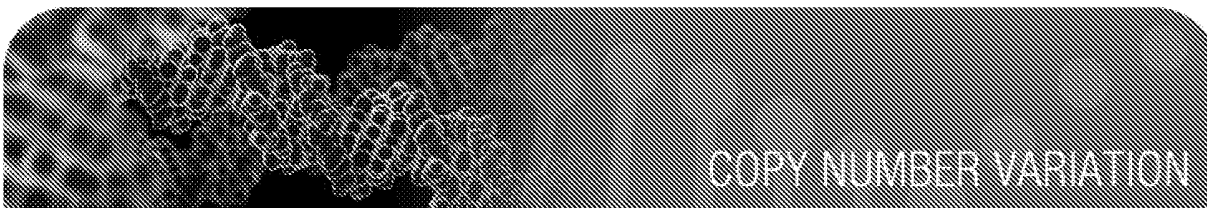
partitioning assembly, the thermal incubation assembly, and the detection assembly, and (ii) to determine a quantity representative of as level of at least one type of target in the sample.

5 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
10 disclosed herein.

Abstract

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

APPENDIX A



QuantaLife Droplet Digital™ PCR Quick Reference

Copy Number Variation Protocol

Purpose

This Droplet Digital™ PCR protocol provides instructions for performing copy number variation experiments using TaqMan® assays designed for a specific target allele and a reference allele. It is important to perform the digestion of the template to ensure the most accurate results. For example, secondary structures in the template may affect accessibility of the polymerase to amplify the target or reference correctly.

Required Materials

Materials and Equipment	Exemplary Source
QuantaLife Droplet Generator	QuantaLife
QuantaLife Droplet Reader	QuantaLife
Thermal Cycler	User provided
QuantaLife 2x Master Mix	QuantaLife
QuantaLife DG8™ cartridges	QuantaLife
QuantaLife DG8™ cartridge holder	QuantaLife
QuantaLife DG8™ gaskets	QuantaLife
ddPCR™ Droplet Generation Oil	QuantaLife
ddPCR™ Droplet Reader Oil	QuantaLife
2x ddPCR™ Buffer Control	QuantaLife
20x TaqMan® target assay	Applied Biosystems
20x TaqMan® reference assay	Applied Biosystems
DNA Template	User Provided
Nuclease free water	Major supplier
Incubator or water bath	Major supplier
Centrifuge	Major supplier
96 well PCR plates	Major supplier
PCR plate heat sealer	Major supplier
PCR plate heat seals	Major supplier
Reagents trough	Major supplier
Standard laboratory supplies: gloves, tubes, pipets, pipet tips, restriction enzymes, ect.	Major supplier

WorkFlow

A Selecting Restriction Enzymes

1. Use bioinformatics tools to select appropriate restriction enzymes which cut around, but not within, the amplicons from the user's assays

B Digest the Template

1. Quantify DNA via absolute quantification with ddPCR™
2. Perform restriction digestion to fragment genome

C Prepare the Master Mix

1. Prepare ddPCR™ master mix

D Generate Droplets

1. Generate droplets with DG8™ cartridge
2. Seal plate
3. Thermal Cycle

E Read Droplets

1. Load plate on reader
2. Create plate map with software
3. Read Plate

F Data Analysis

1. Analyze data using QuantaSoft™ analysis program

A

Selecting Restriction Enzymes

1. Use a website with a genomic search tool, such as the National Center for Biotechnology Information (NCBI). Search the nucleotide data base to find the nucleotide sequence of the gene of interest. Also obtain the sequence surrounding the gene of interest, for example, up to about 500, 1000, or 2000 base pairs on both sides of the gene of interest.
2. Copy the sequence and surrounding sequence for the gene of interest and paste it into another (second) web browser page that is capable of aligning a preselected assay (e.g., the forward and reverse primers) with the sequence and the surrounding sequence for the gene of interest. This will allow the determination of where the assay amplicon will reside in the gene sequence and surrounding sequence. Suitable online tools can be found at NCBI's BLAST align two sequences tool.
3. Important! The goal is to select restriction enzymes to cut around the amplicon, but not within the amplicon as cutting within the amplicon would render the assay useless.
4. In a third web browser window, open a tool to find restriction enzymes for the gene sequence and the surrounding areas. Suitable tools can be found on New England Biolabs website. Paste the sequence and surrounding sequence for the gene of interest into the enzyme finder tool, and select enzymes that cut around the amplicon using the knowledge of where the amplicon resides in step 2 above.
5. It may be necessary to perform a double digestion depending on the sequence of amplicon and surrounding sequences.
6. Important! Repeat steps 1-5 for the reference assay and ensure that the restriction enzymes selected for the target gene do not cut the amplicon of the reference gene and vice versa.

B

Digest the Template

1. Quantify the DNA (e.g., by using the QuantaLife absolute quantification protocol and/or other suitable technique).
2. Thaw the template on ice.
3. Use the knowledge gained from previous section on selecting restriction enzymes in choosing the proper digest to perform.

4. A desired working range of template in the final 1x reaction for CNV analysis is between 100 and 1500 copies per μL . For human DNA, approximately 3.3pg is equivalent to 1 genomic copy. This may need to be adjusted if the target is expected to have very high copy numbers. This is necessary to avoid saturating every droplet with the target, which would make every droplet positive.
5. Set up a restriction digest according to restriction enzyme manufacturer's instructions and follow manufacturer's guidelines on how much enzyme to use with respect to the DNA weight. A typical digest scheme is shown in table 1 below.
6. Verify digestion reaction is complete with a suitable technique (e.g., gel electrophoresis), or use a control template and assay that cuts the amplicon and should not give a positive result. A positive control of undigested template can also be used (optional).

Table 1

Template Digestion	Final conc.	1 rxn (μL)
10x Digest Buffer	1x	5
10x BSA	1x	5
Restriction Enzyme		1
DNA Template (25ng/ μL)	16.1 ng/ μL	32.2
Water	q.s.	6.8
Final Volume (μL)		50

Important Considerations for Template Preparation

- Select restriction enzymes that do not cut either the target or reference amplicons.
- The restriction digest DNA should be at a higher concentration for later dilution in the master mix.
- If possible, calculate how many copies are expected per genome and ensure that every droplet will not be oversaturated with multiple copies in each droplet by lowering the concentration of template.

- QuantaLife ddPCR™ Master Mix makes use of dUTPs; therefore, the user can use UNG to decrease the likelihood of carryover contamination of amplicons.

C

Prepare the Master Mix

1. Thaw ddPCR™ 2x Master Mix and 20x primer/probe TaqMan® assays on ice.
2. Calculate how many replicates are necessary for experimental design, including “No Template Controls” (NTCs) and positive controls.
3. Assemble 1x master mix according to experimental design; table 2 presents a typical scheme with sufficient master mix for generating 4 replicate wells on a chip.
4. After adding all components of the ddPCR™ reaction, mix thoroughly using a suitable technique (such as by vortexing).

Table 2

1x Master Mix Formulations	Final conc.	1 rxn (µL)	5 rxns
2x ddPCR™ Master Mix	1x	10	50
20x TaqMan® Target primers/probe (FAM)	1x	1	5
20x TaqMan® Reference primers/probe (VIC)	1x	1	5
DNA Template Digested (16.1 ng/µL Table 1)	1000 copies/µL	4.1	20.5
Water		3.9	19.5
Final Volume (µL)		20	100

D

Generate Droplets

1. Use a DG8™ cartridge to generate droplets. One cartridge makes 8 wells; therefore, it may be advantageous if making replicate wells to generate 4 replicates of two different samples per cartridge (8 wells total).
2. All wells must contain a liquid being either 1x ddPCR™ master mix or 1x ddPCR™ Buffer Control for optimal droplet generation. Do not use water only.
3. Once all wells are loaded in the proper aqueous wells of the cartridge, add ddPCR™ Droplet Generation Oil to the oil wells on the cartridge. A reagent trough in combination with a multichannel pipet may be helpful to dispense the oil into the oil wells on the cartridge.
4. After the aqueous and oil wells are all full of their respective liquids, place the cartridge into the cartridge holder, position a gasket over the tabs on the holder and place the entire assembly into the Droplet Generator instrument.
5. Use a multichannel pipet for optimal efficiency to transfer the droplets from the cartridge to a 96 well PCR plate.
6. Repeat the droplet generation (steps 1-5) process until the experimental design is complete.
7. Seal the PCR Plate with a foil seal and a PCR plate heat sealer.
8. Insert the PCR plate into the user's thermal cycler and thermal cycle the plate. Table 3 presents general two-step thermal cycle conditions typically used, however, each assay should be optimized for best performance.

Table 3

Thermal Cycling Parameters	Cycles	Duration
95°C	1	10 min
94°C melt and 60°C anneal/extend	40	30sec (melt) 1min (anneal/extend)
98°C Heat inactivation	1	10 min

Important Considerations for Master Mix Preparation and Droplet Generation

- Each DG8™ cartridge generates 8 wells of droplets.
- Any extra wells on the cartridge must be filled with 1x ddPCR™ Buffer Control.
- It may also be advantageous for the user to design their experimental plate layout on a 96 well plate in columns, as the cartridge contains 8 wells.
- 1x QuantaLife ddPCR™ master mix must be used for proper droplet formation and for extra wells on the cartridge, 1x QuantaLife ddPCR™ Buffer Control must be used for proper cartridge function in the droplet generation process.
- A thermal gradient optimization is recommended for the TaqMan® primers and probes prior to running a CNV experiment in order to maximize the thermal cycling conditions to give the best performance for the user's assay.

E

Read Droplets

1. **After thermal cycling, load the sealed 96 well plate inside the Droplet Reader instrument plate holder.**
2. **On the Droplet Reader computer, open QuantaSoft™ and design a new plate with a layout according to the user's experimental design. Importantly, designate the experiment type as "CNV" in the well editor feature during plate setup.**
3. **Designate which assay is the Target and which is the Reference assay in the correct color channel (FAM or VIC) in the well editor feature. Detailed instructions on QuantaSoft™ new plate setup can be found in the user guide.**
4. **Prior to running the plate, the user can select in the plate setup whether the instrument acquires data sequentially in rows or columns. The default is columns.**
5. **Once the plate layout is complete, select "Run" to begin the droplet reading process.**

Important Considerations for Droplet Reading

- Ensure there is enough Droplet Reader Oil in the instrument and that the waste is empty prior to a run if a message in QuantaSoft™ product indicates that the instrument needs this service performed.

- Prior to a run, the instrument can be set to interrogate droplets either in rows or columns.
- Designate “CNV” for the experiment type in the well editor (for performing a copy number variation assay).
- Indicate which assay is the target and which assay is the reference in the well editor on in the QuantaSoft™ product during setup.

F

Analyze Data

1. After the Droplet Reader has finished interrogating all wells, QuantaSoft product can be used to analyze the data in each well. Select the analyze button on QuantaSoft™ product to begin the analysis.
2. If the plate was set up correctly for CNV analysis, the CNV tab will appear. The user can always go back to the setup post data collection and edit each well to change the experiment type and designate which assay is the reference and which is the target.
3. Remember the default for the reference copy number for a CNV type experiment is 2 (diploid). This can be changed by editing the experiment in the setup.
4. Select a well the user wishes to analyze, ensure that the thresholds are set appropriately by viewing the data in FAM vs. VIC, and then select the CNV tab to view the CNV value for that well. Multiple wells can be viewed by selecting more wells.
5. Detailed instructions for how to interpret the data can be found in the user manual.

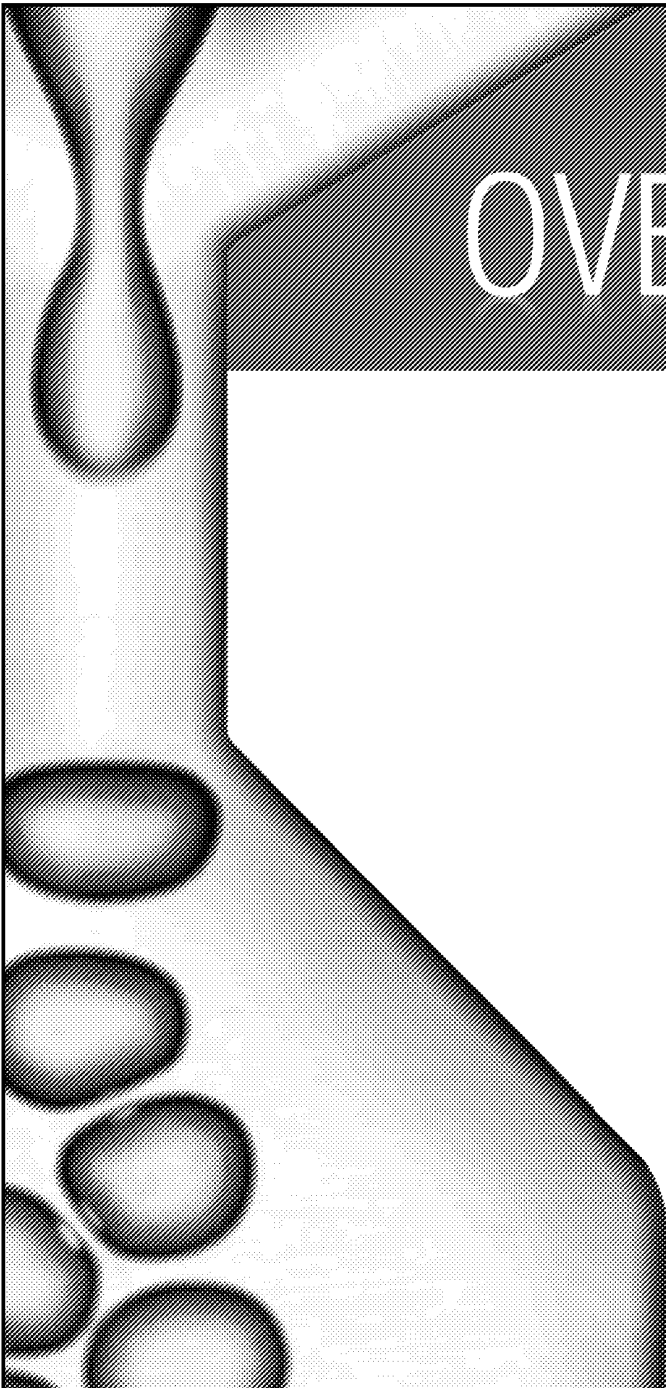
Important Considerations for Data Analysis

- If no channel was selected as the reference assay, the default is the VIC channel for the reference assay and the FAM channel for the target in subsequent CNV calculations during analysis.
- The CNV experiment (in setup) allows the user to set the number of copies for the reference, the default is 2. This can be changed by editing the experiment in QuantaSoft™.

Recommended Human Reference Assays

Assay	Chromosome	Forward Primer	Reverse Primer	Probe
QL_RPP30_1	10	GATTTGGACCTGCGAGCG	GCGGCTGTCTCCACAAGT	5'-VIC-CTGACCTGAAGGCTCT-MGB-3'
QL_uc44_ch1	1	TGAGGGATTTCGGCAGATGTTG	CTGAAAGGCTGGACTTGACAGA	5'-VIC-ACTGTGTGCTGGACCT-MGB-3'
QL_uc160_ch5	5	GCTGAACTGAGCGGAATTGGA	CCCAGCCTCATCTCTCATACG	5'-VIC-TCGCTGACCTTCCCTC-MGB-3'
QL_uc197_ch6	6	TTTCAGCCAAGCATACTCCATCAT	CGACTGGCTGCACAGC	5'-VIC-TTGATGCATAAATTCA-MGB-3'
QL_uc377_ch14	14	GGTTGTGATGATGATGGCTTGT	GGCAGGTTAGAGTCAAAGGTTTCC	5'-VIC-CCTGAGCCATACCCAGATA-MGB-3'
QL_uc406_ch16	16	CATGAGCACCAAGTTCCTACAA TGAT	TGCTTTGGATTTCGTTTTCGTGATT	5'-VIC-ACGAGGTACCTCAGTGT-MGB-3'
QL_uc412_ch17	17	GGA CTGAGCCAATGGTAGA C	GCAAGGTGTCTCAGAACCCAA TT	5'-VIC-ATTGCAACAACTGG-MGB-3'
QL_uc473_chX	X	GGGAACAGAACAAAGCTGGTCTT	GCAAGACCCTGTGTAAGAACTTTGA	5'-VIC-CTATCACATGTGGATCGTA-MGB-3'

APPENDIX B

A grayscale image of a microfluidic chip. On the left, a vertical channel contains a series of droplets. A single droplet is shown in the process of being dispensed from a narrow opening at the top. The background is a dark, textured gray.

OVERVIEW

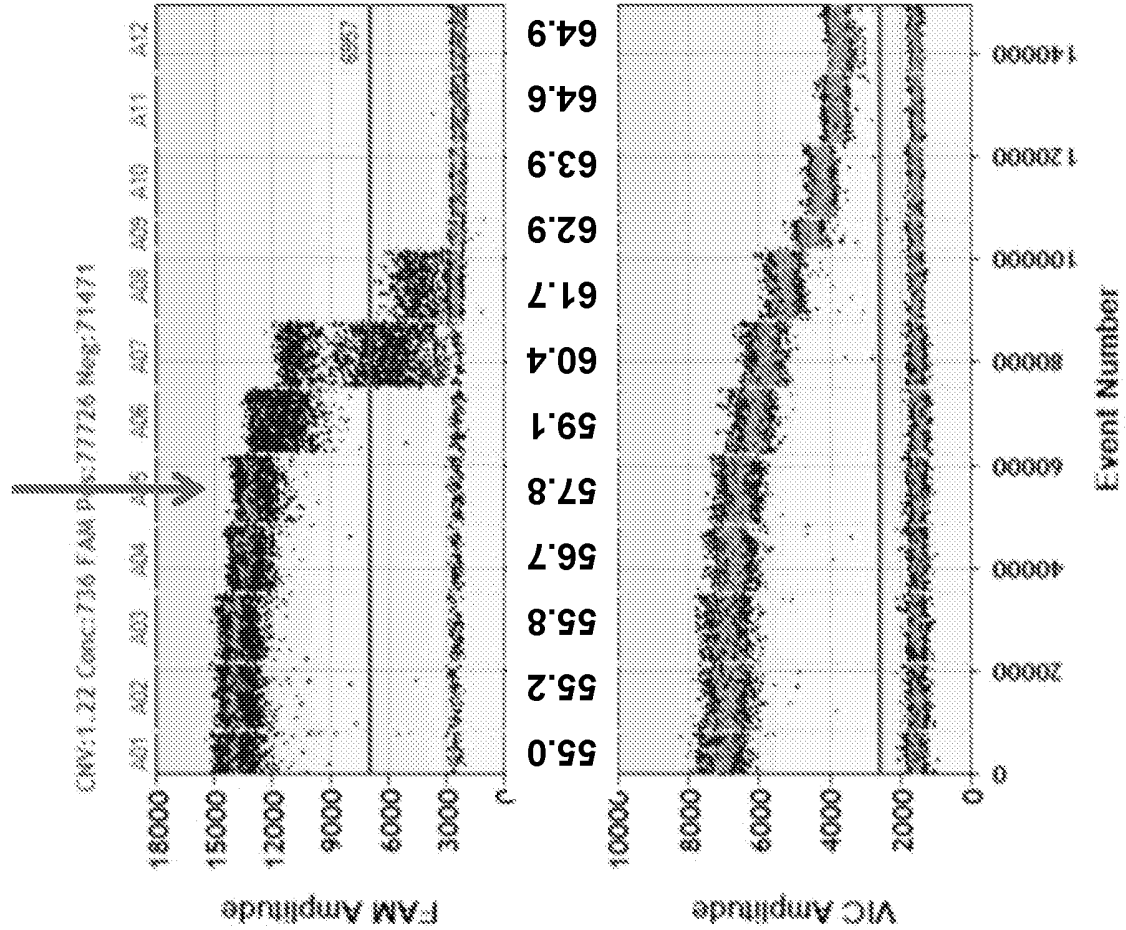
CNV Assays

- CR1 –FAM (NA19204 – Mse I digested) 4-6 copies common
- C4A - VIC (NA18502 – Alu I digested) 0 – 5 copies common
- C4L – VIC (NA19239 – Alu I digested) 0 – 5 copies common
- C61 – VIC (NA 19239 – Alu I digested) 2 – 5 copies common
- C63 – VIC (NA 19239 – Alu I digested) 2 – 5 copies common

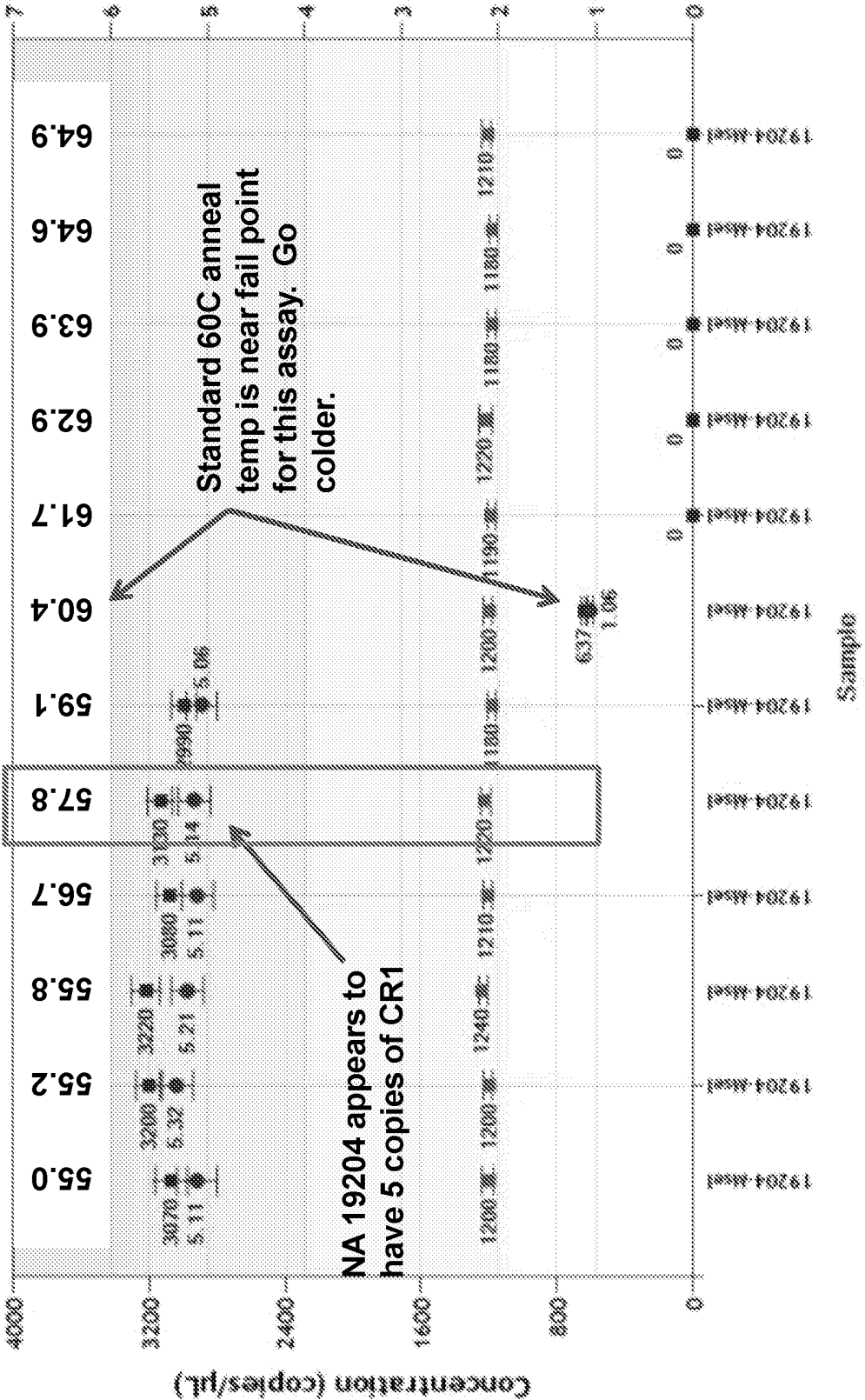
SNP Assays

- Rs2273863 (LGALS8) – (NA18502 – Alu I digested)
- Rs273259 (IF144L) – (NA 19239 – Alu I digested)
- Rs216008 (CACNA1C) – (NA18502 – CviQI digested)

CR1 - FAM

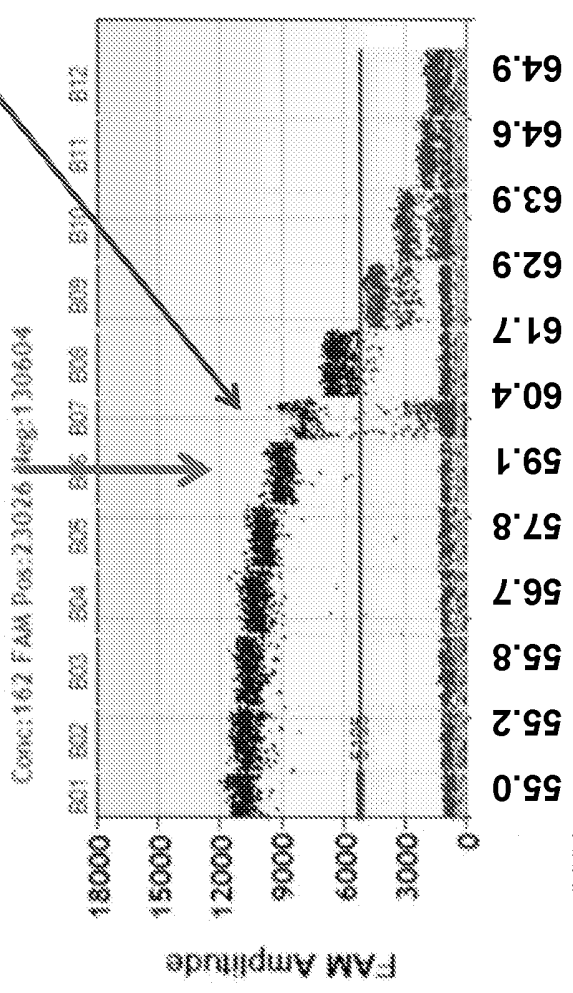


CR1 - FAM



C4A - VIC

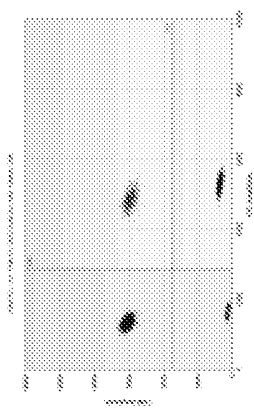
Clog may have shredded droplets.



RPP30

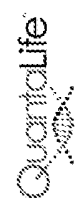
RPP30

59.1

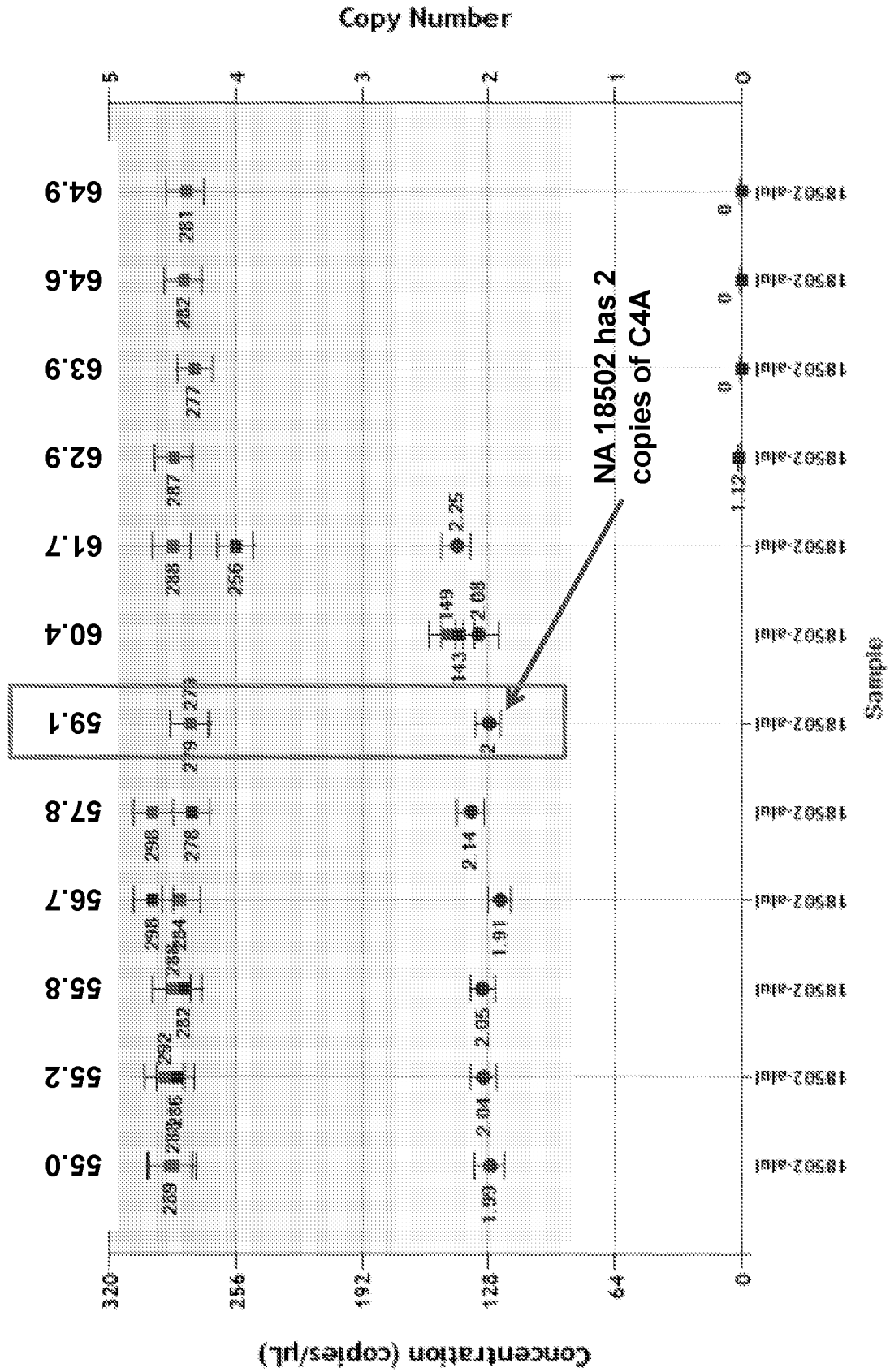


C4A

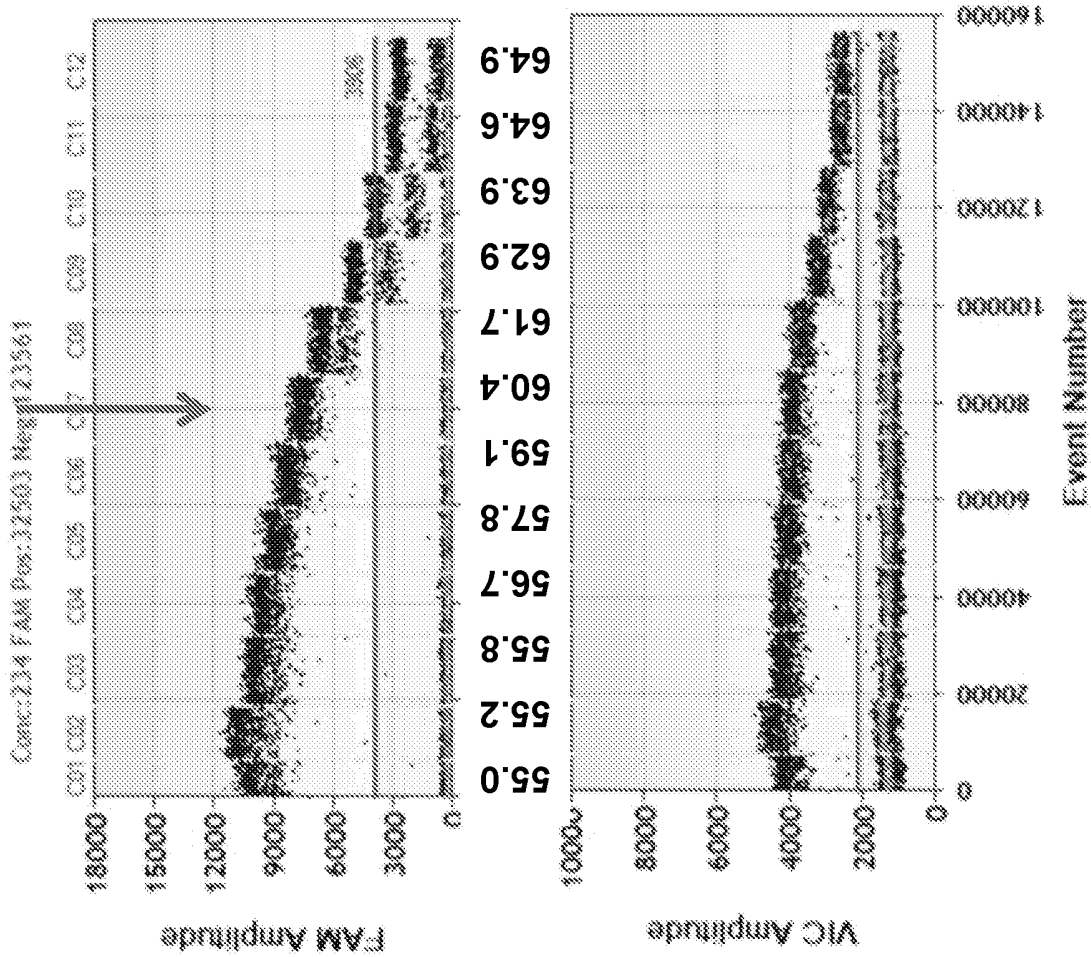
C4A



C4A - VIC

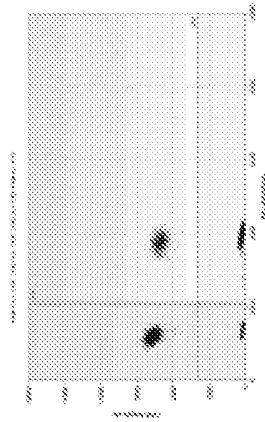


C4L - VIC



59.1 – 60.4 appear good

60.4 shown below



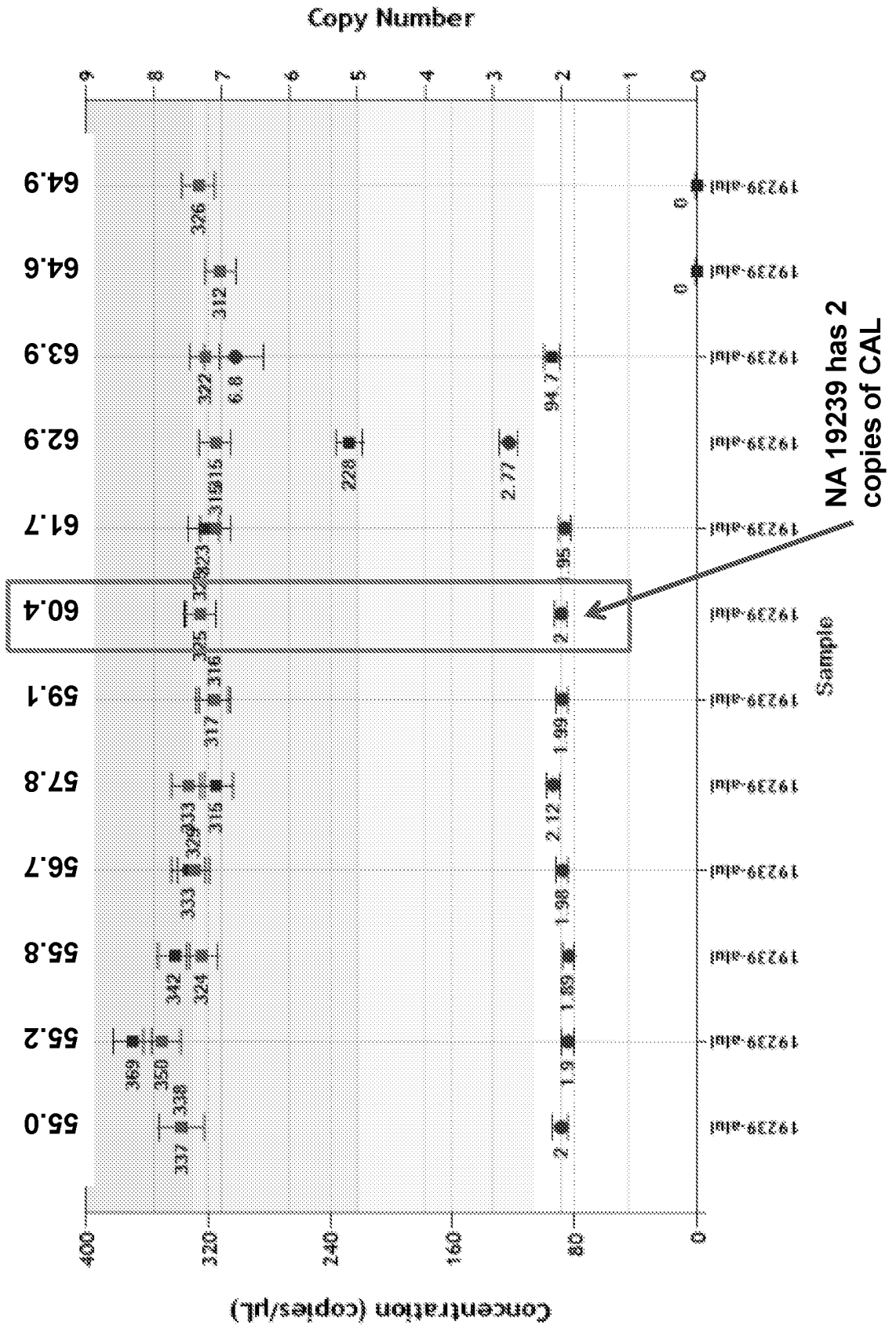
RPP30

C4L

C4L

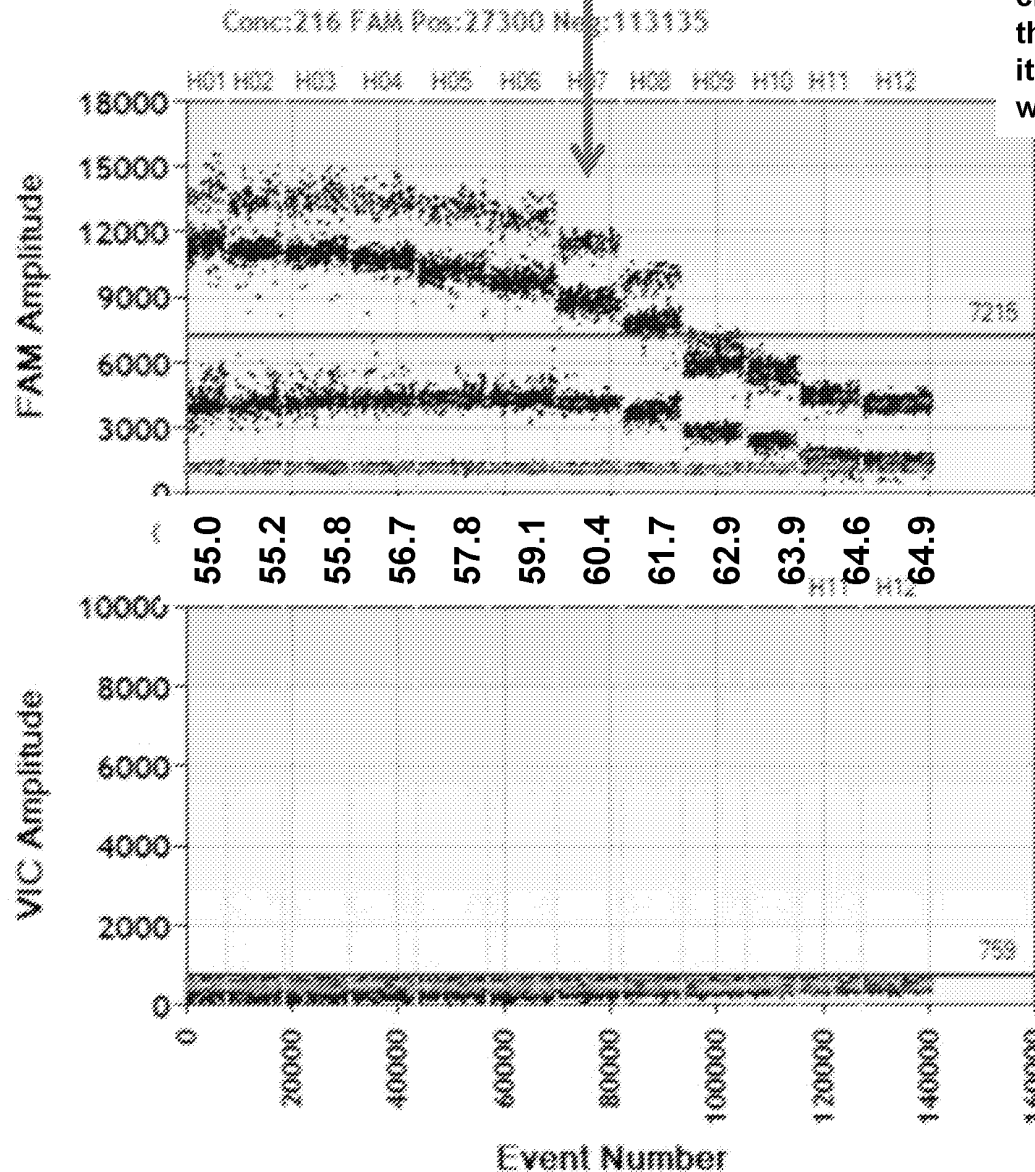
Quantalife

C4L - VIC



C61 - VIC

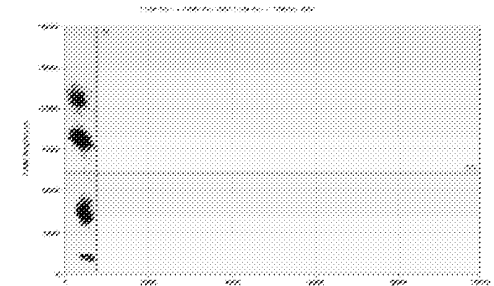
According to the provided sequences, this assay is a VIC assay, however there is no signal in the VIC channel. After looking at these curves, I looked at the color of the probe provided for this assay, and it looks to be the shade of a FAM dye. So I believe we are seeing both assays in the same channel.



59.1 shown below

RPP30

RPP30



C61

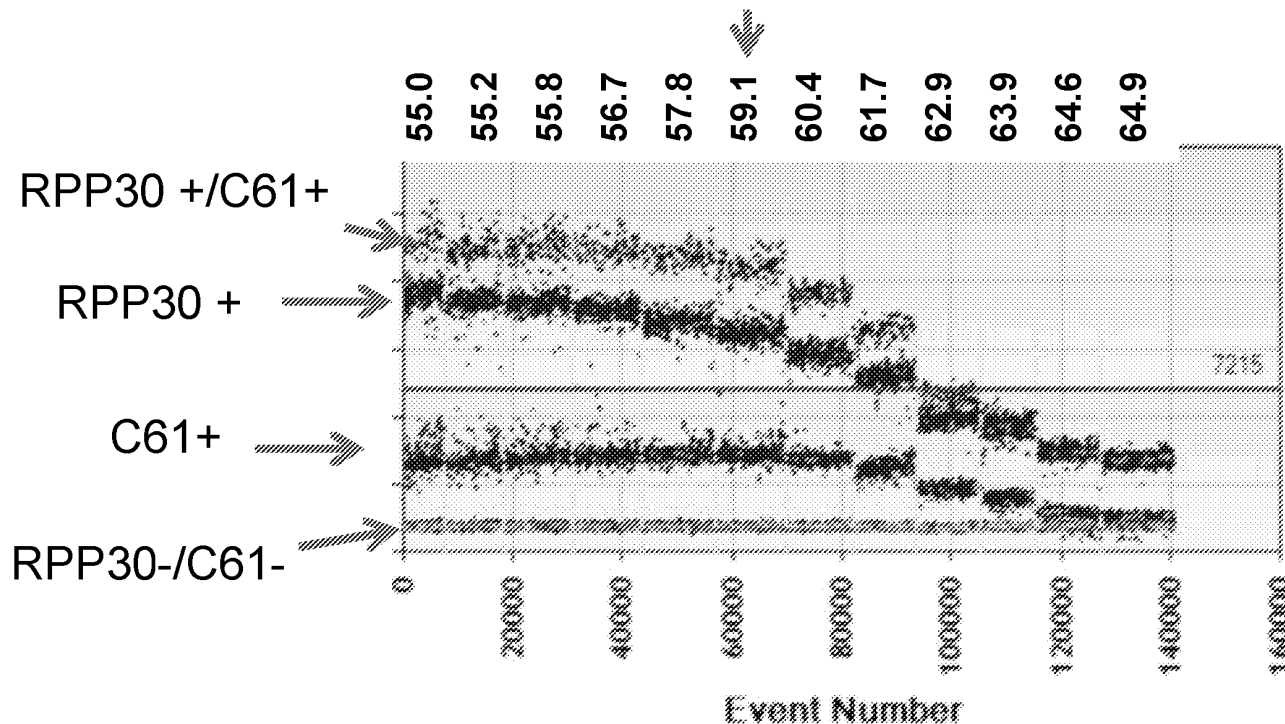
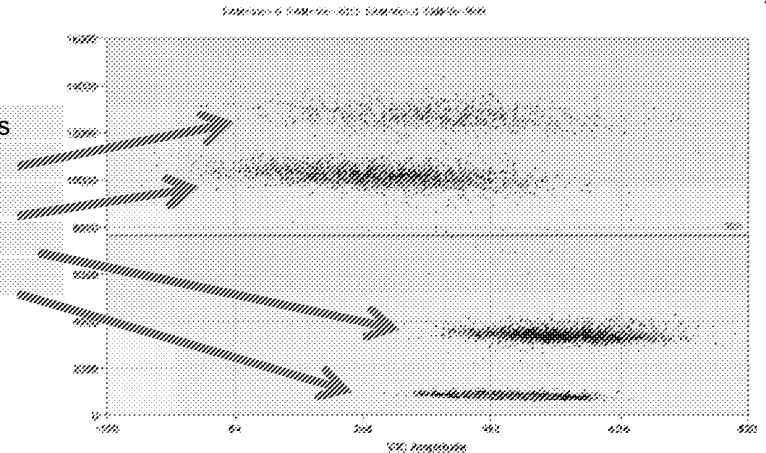
C61

Ideally, this experiment should be repeated with a RPP30-VIC reference assay and the CNV values examined. Without repeating it, it appears as though 59.1 will work well.

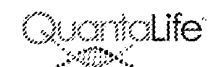
See next slide for more info.

C61 – VIC: Identifying populations

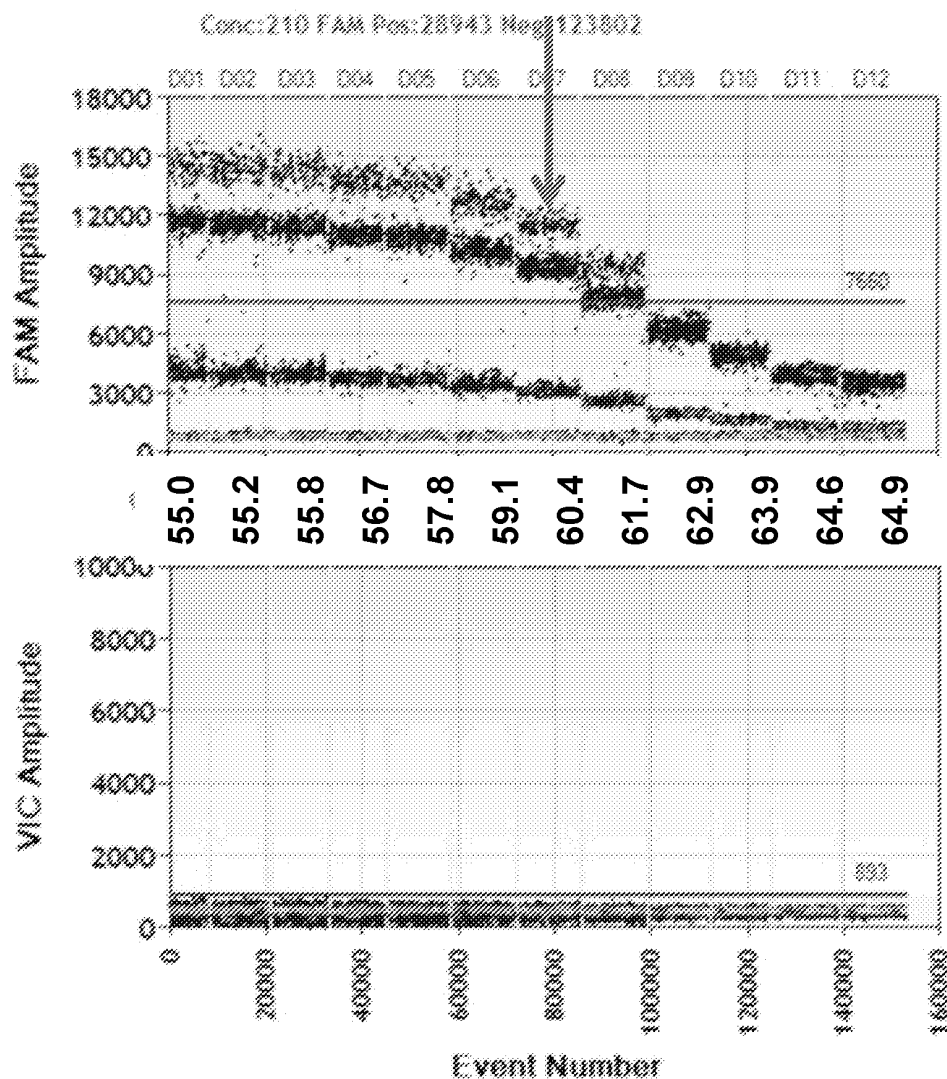
	# droplets
RPP30+/C61+	1168
RPP30+	2865
C61+	2854
RPP30-/C61-	6782



The selection tool was used to draw boxes around these clusters and count the number of droplets in each population. There are 4033 RPP30+ drops, 4022 C61+ drops, and 6782 negative drops. Therefore, this sample appears to have a CNV = 2.



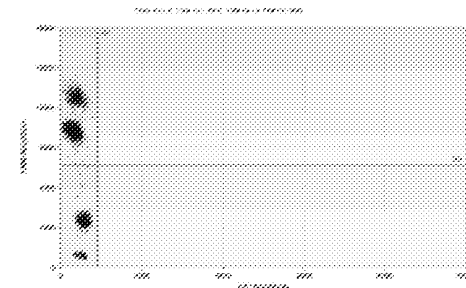
C63 - VIC



Same story as C61.
C63 is a FAM labeled probe.

RPP30

RPP30



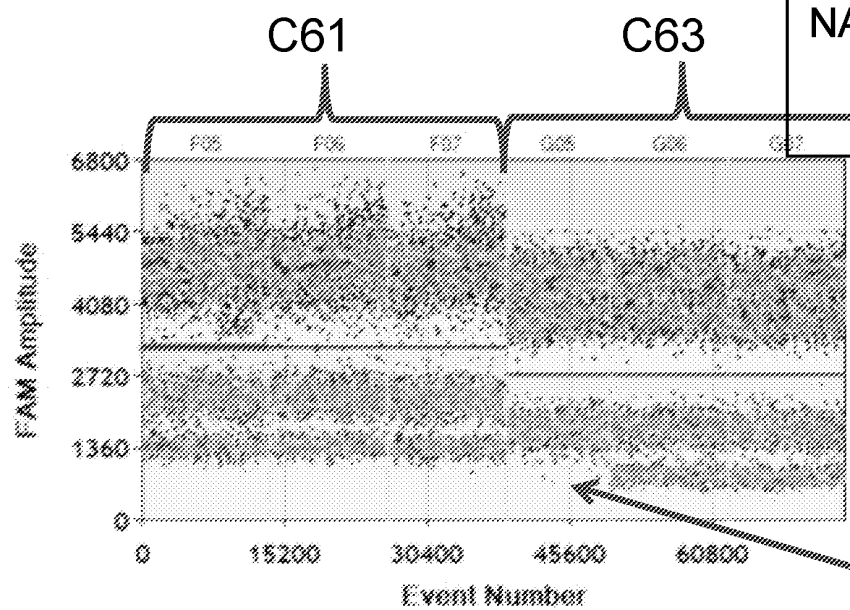
C63

C63

There are 3552 RPP30 positive drops, 3441 C63 positive drops, and 6224 negative drops. CNV = 2.

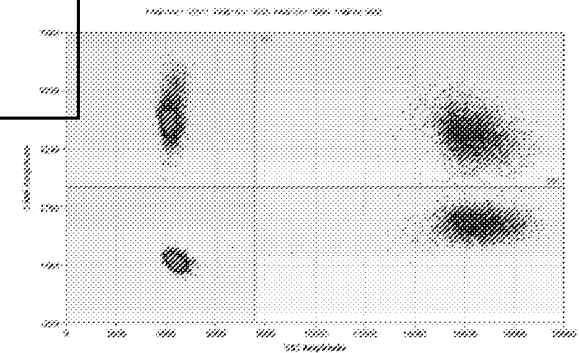
Solution: Same as for C61.

Follow-up for C61 and C63 Pair with RPP30-VIC rather than RPP30-FAM



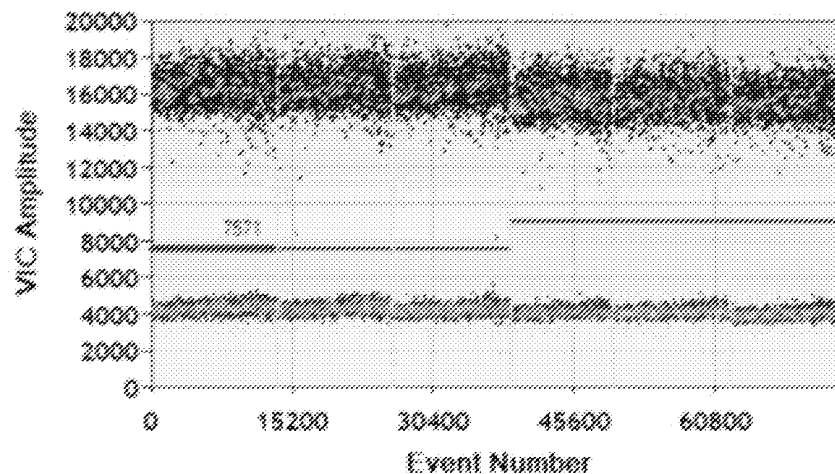
C61

Double negatives were dropped in this well. However, assuming that such occurrences are infrequent, they may be overcome using replicates.

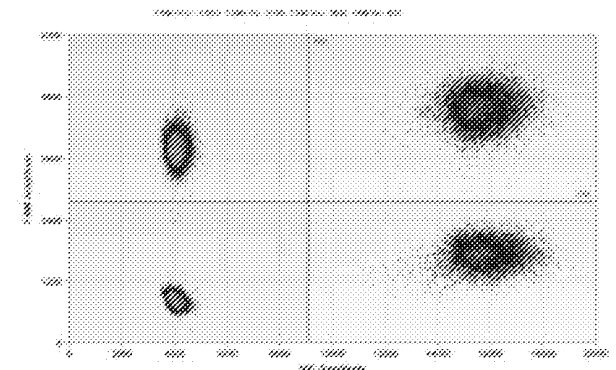


RPP30

RPP30 – VIC

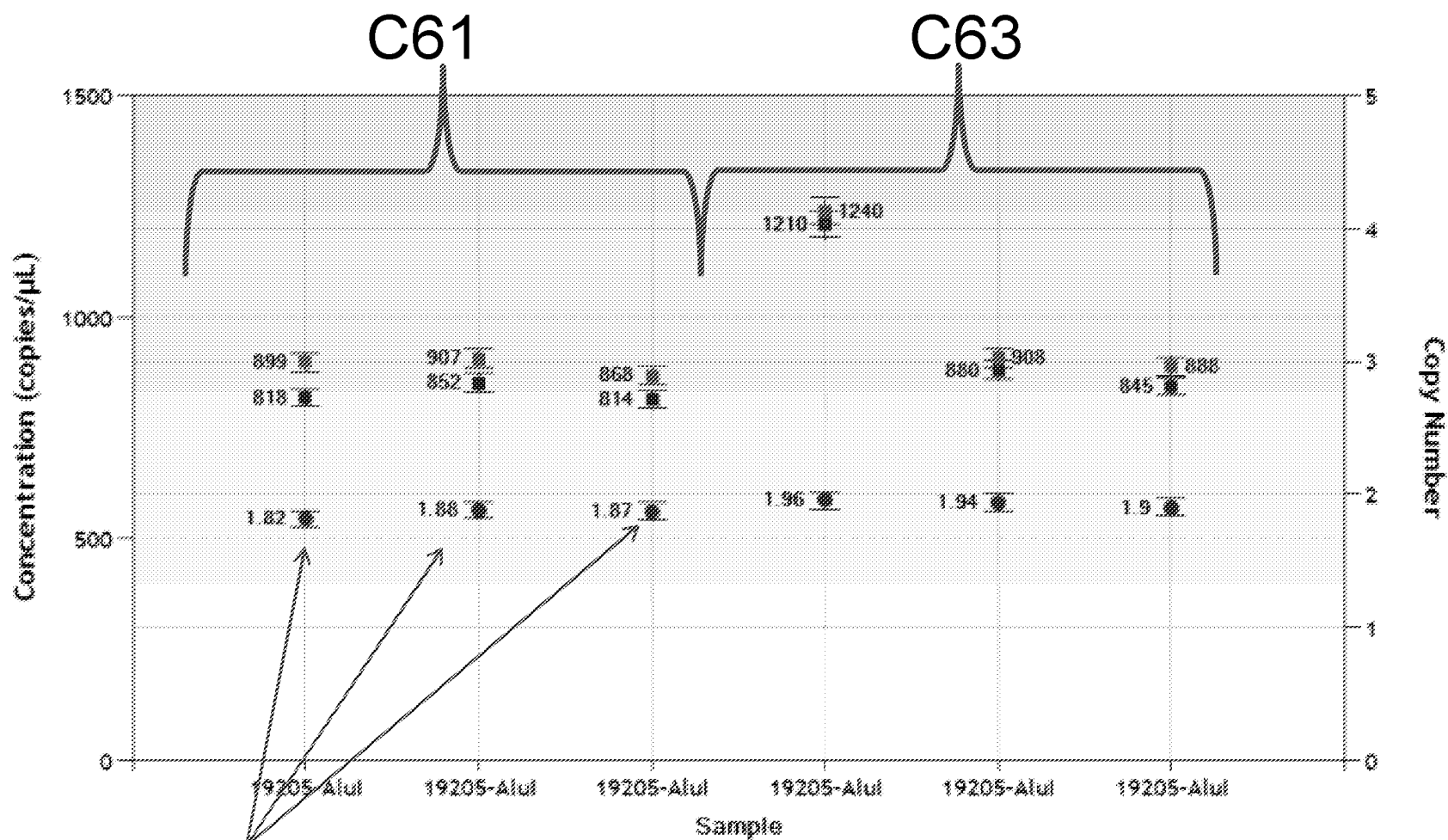


C63



RPP30 *antaLife*

C61 & 63 – Triplicate Measurements



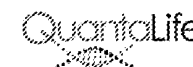
Slightly off-integer values. This may be sample specific.
Alternatively, or in addition, selecting a different reference assay
and/or different restriction enzyme (RE) may improve these values.

SNP assays

Samples that are heterozygous for the loci of the provided assays were used to test these assays.

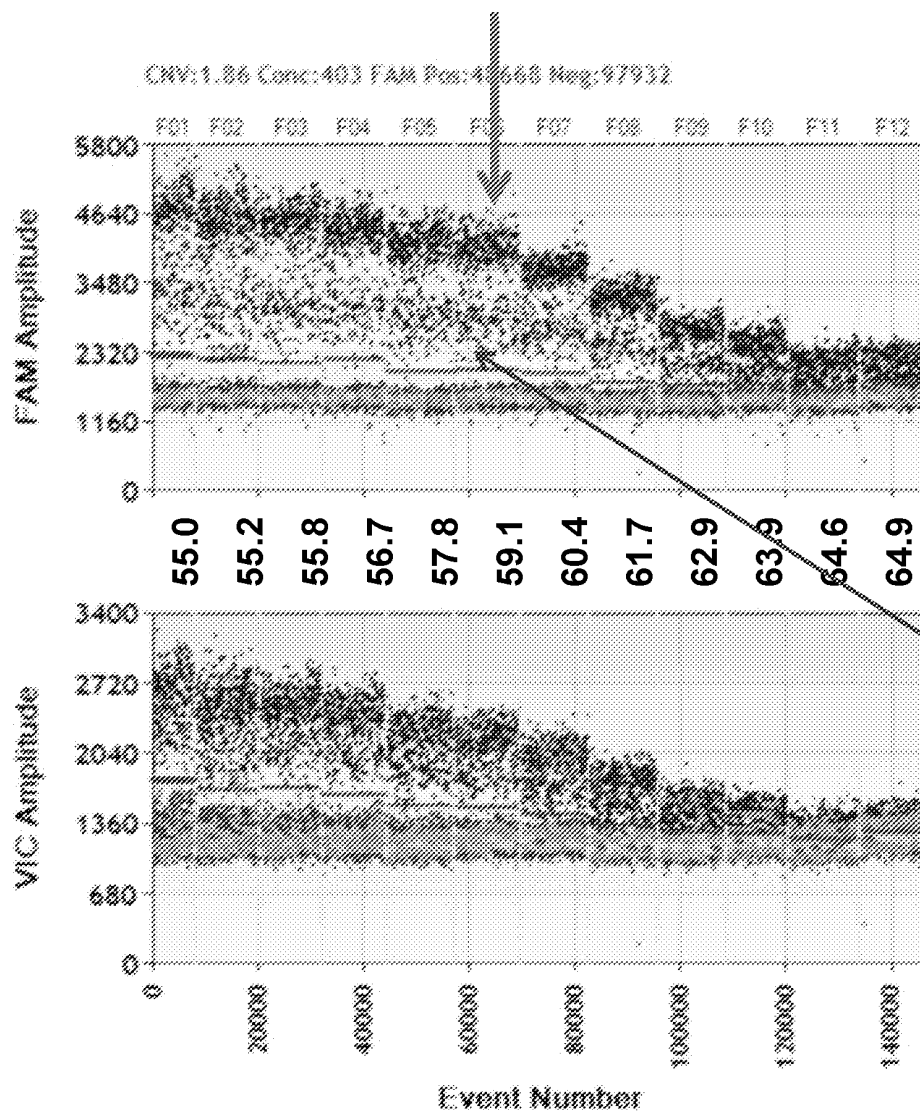
Testing alternatively may be performed using a mixture of homozygous samples of opposite genotypes; however, interpretation may be more complicated if the ratio of genotypes deviates from 50/50.

The sequence surrounding the SNP in a heterozygous sample may be checked to determine which restriction enzymes should be avoided due to their potential to cut the amplicon (which may be of unknown size).



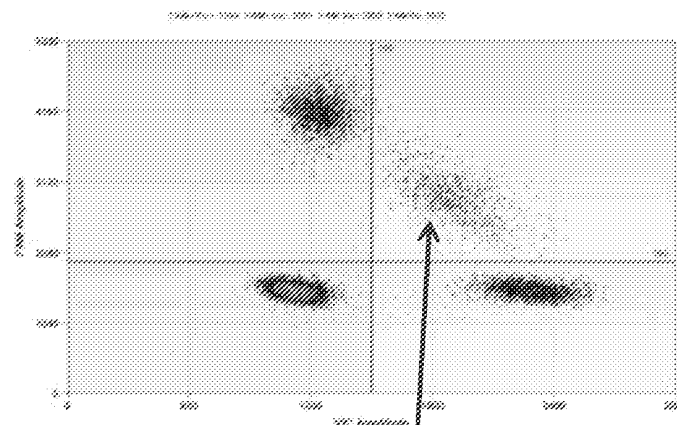
Rs2273863 (LGALS8)

(NA 18502 – Alu I digested)
T/G (FAM/VIC)



T allele

59.1

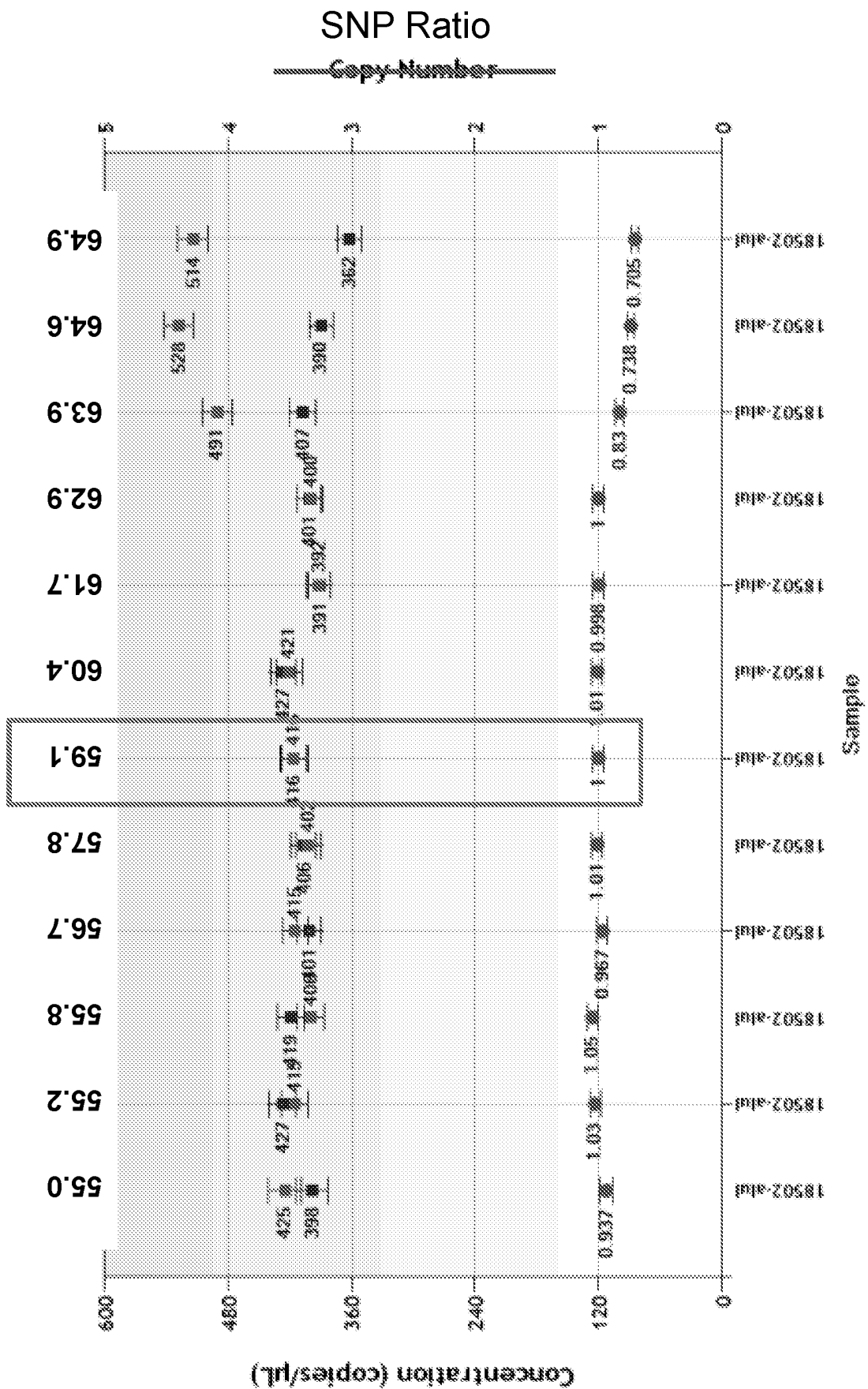


G allele

The fluorescence in double positive droplets is less than that seen in single positive droplets due to competition. The reduced fluorescence is manifest as a double band in the FAM VIC plots shown on the left.

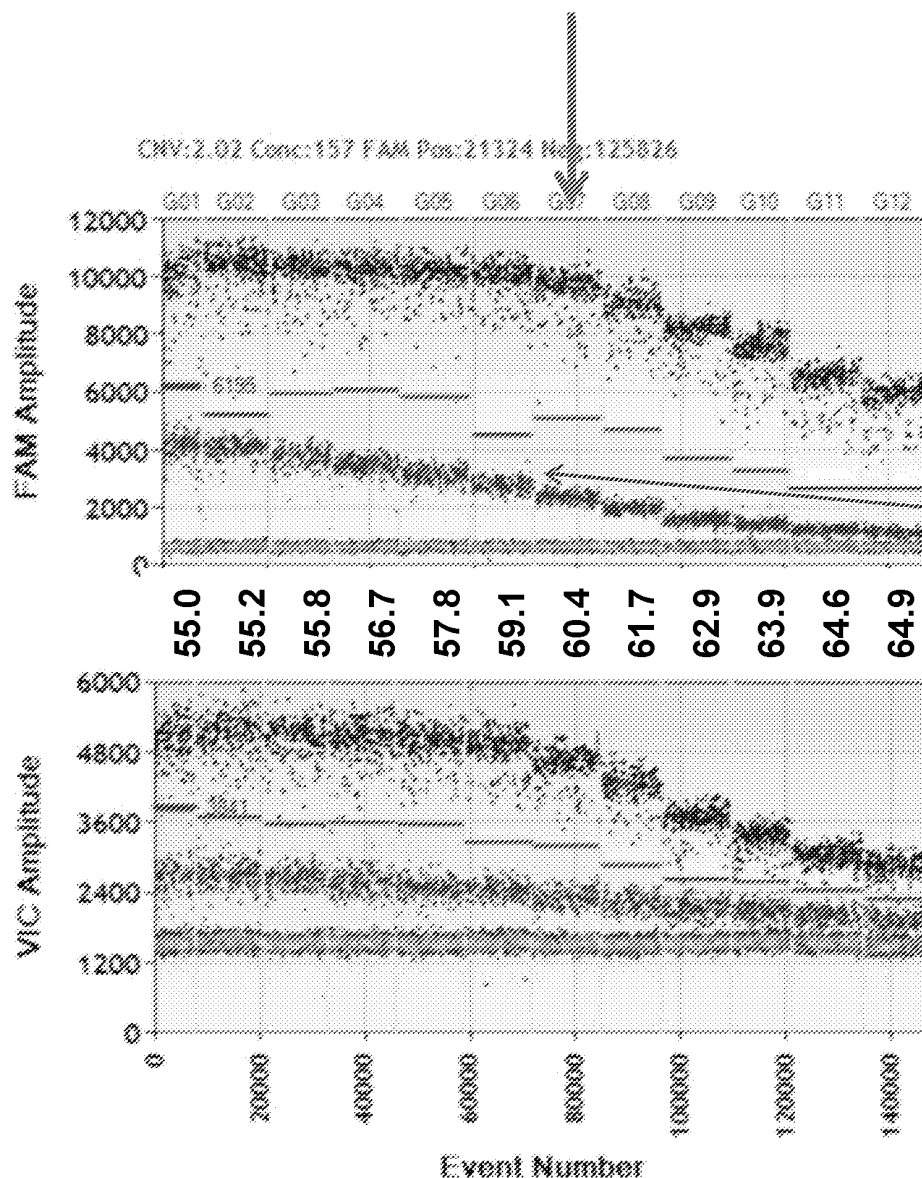
On SNP assays, tendency should be to pick the warmest temperature that gives good separation in both channels with a small buffer to account for thermal-cycler differences.

Rs2273863 (LGALS8)

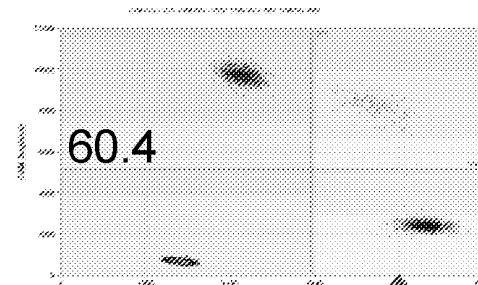


Rs273259 (IF144L) –

(NA 19239 – Alu I digested)
G/A (FAM/VIC) SNP assay



G allele

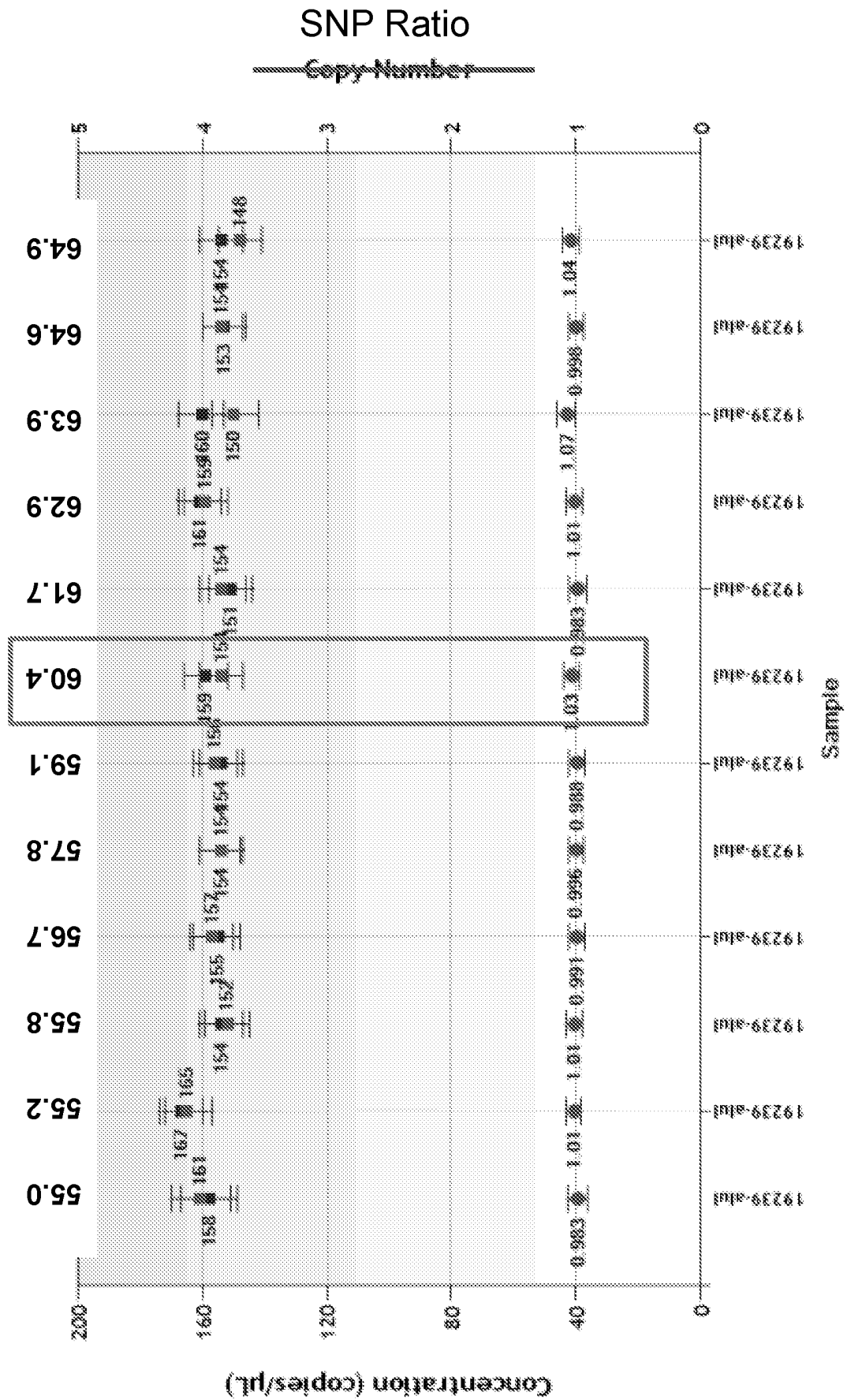


A allele

Droplets containing only the 'A' allele have elevated FAM fluorescence, but these droplets are not positive for the 'G' allele. The same is true for the droplets containing only the 'G' allele, as these have higher amounts of VIC fluorescence than the double negative droplets, but do not contain the 'A' allele.

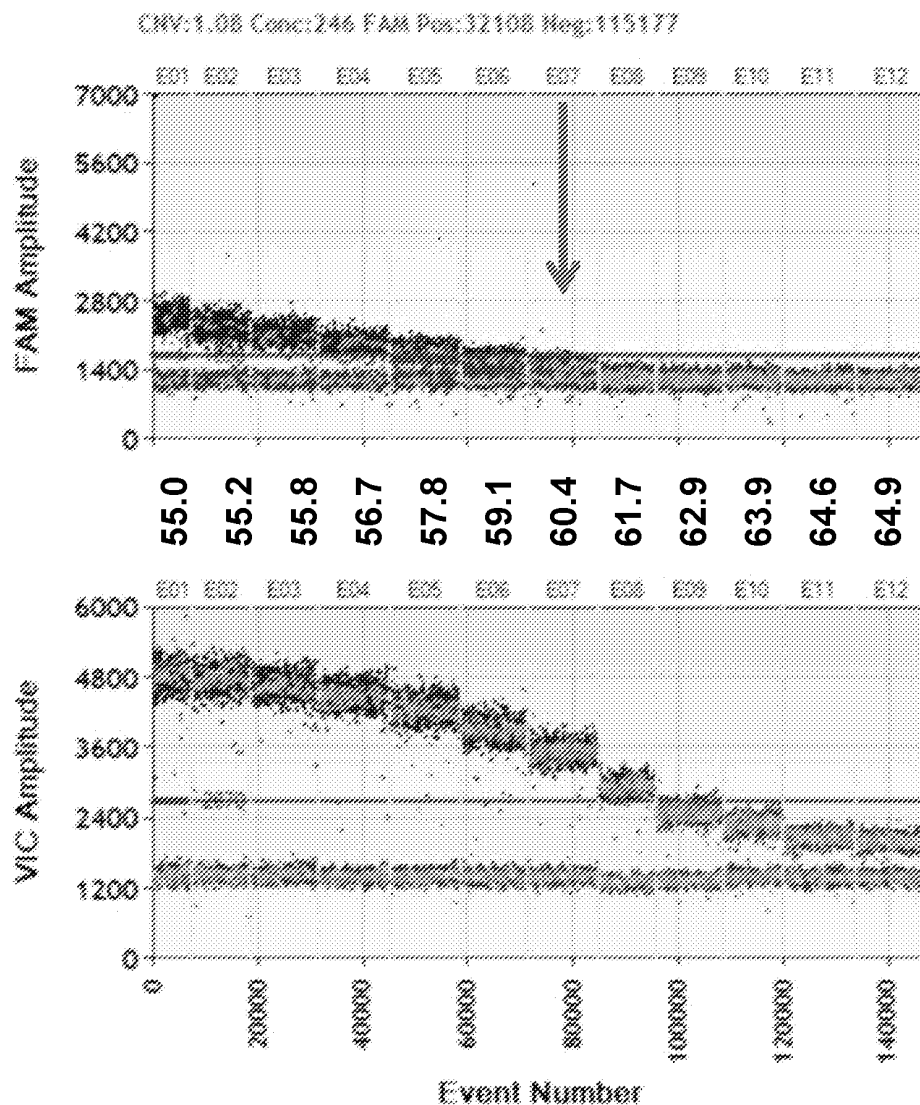
QuantaLife

Rs273259 (IF144L) –



Rs216008 (CACNA1C) –

NA 18502, CviQI cut



Rs216008 is reported to be a T/C (Fam/VIC) SNP assay.

In the absence of a sample known to carry this mutation, experiments were performed using NA18502, which is homozygous (one report says C/C, another says G/G).

According to our data, it appears that this sample is C/C.

As expected, these data show the FAM probe (T) binds to the C allele at colder temperatures.



Follow-up on RS216008 (CACNA1C)

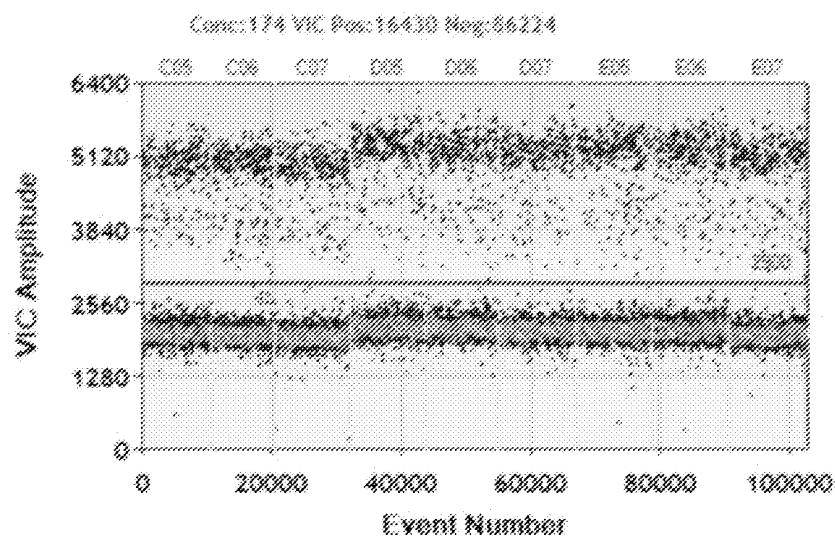
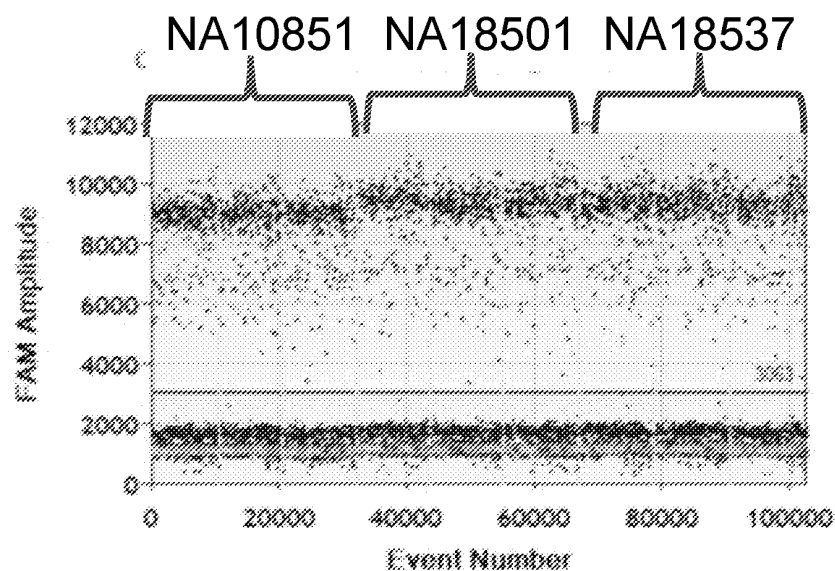
Based on a gradient using NA 18502, an annealing temperature around 60.4C for Rs216008 (CACNA1C) appears to be ideal.

Three samples with ambiguous genotypes for this locus were analyzed (at 60C annealing temp):

<u>Sample:</u>	<u>Genotype</u> (according to Coriell)
NA 10851	C/T or A/G
NA 18501	C/T or A/G
NA 18537	C/T or A/G

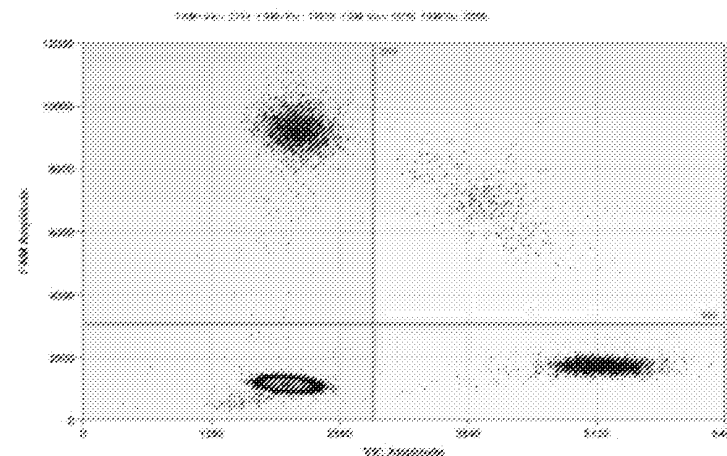


RS216008 (CACNA1C)



Each of these three assays appears to have a C/T genotype - not the alleged A/G genotype by one or more groups reporting to Coriell.

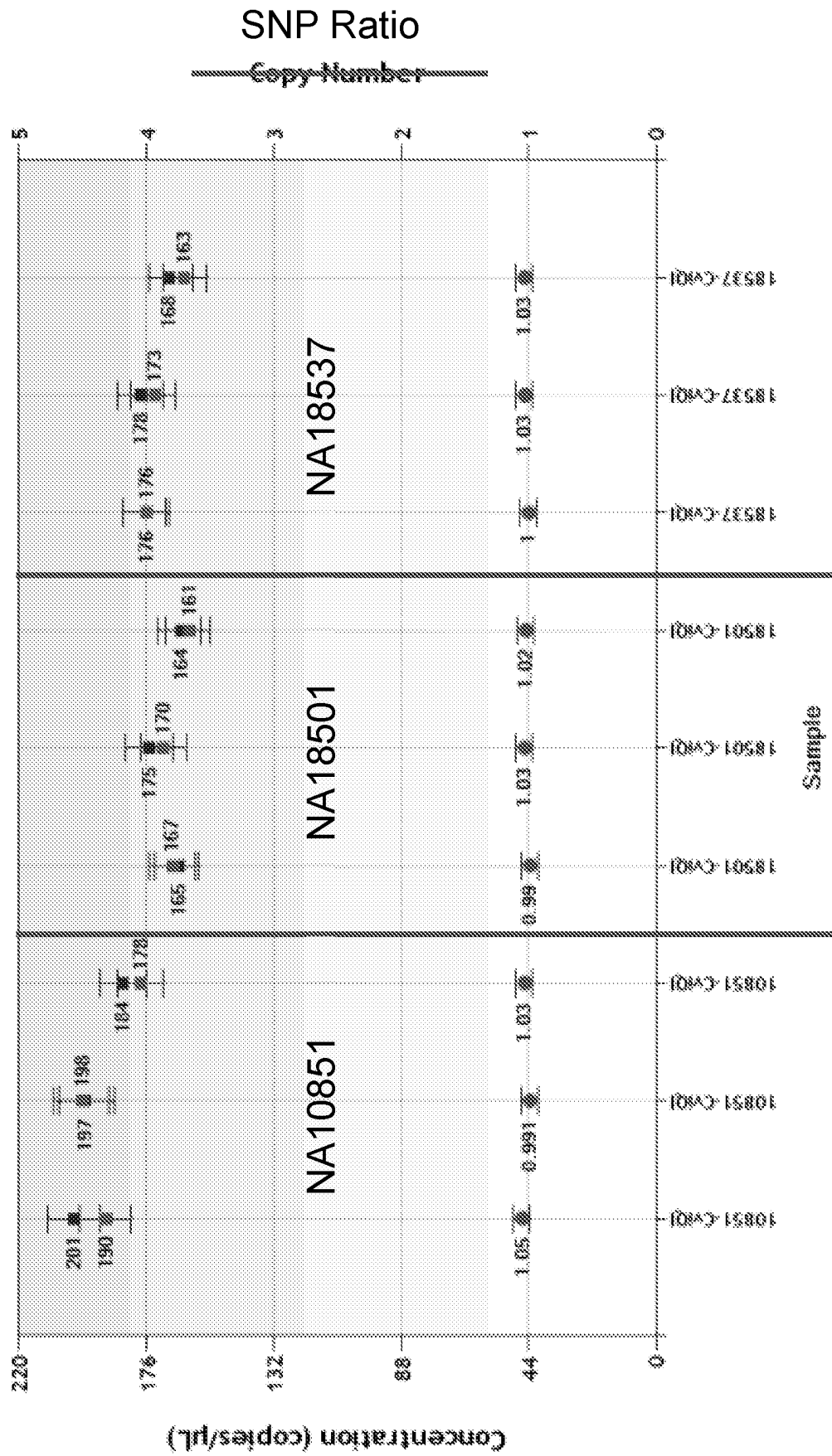
All three shown



QuantaLife

Triplicate Wells - RS216008 (CACNA1C)

ddPCR Concentration / Copy Number Variation



Summary – All assays successfully screened and Ok'ed for use in ddPCR.

CNV Assays

CR1 –FAM: pair with RPP30-VIC, digest with Mse I, anneal at 57.8 (not 60!)

C4A - VIC: pair with RPP30-FAM, digest with Alu I, anneal at 60

C4L – VIC: pair with RPP30-FAM, digest with Alu I, anneal at 60

C61 – **FAM**: pair with RPP30-VIC, digest with Alu I, anneal at 60

C63 – **FAM**: pair with RPP30-VIC, digest with Alu I, anneal at 60

SNP Assays

Rs2273863 (LGALS8): digest with Alu I, anneal at 59.1

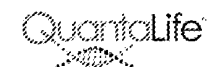
Rs273259 (IF144L): digest with Alu I, anneal at 60.4

Rs216008 (CACNA1C): digest with CviQI, anneal at 60

Probably can be run at 60C without problems.

Optimal annealing temperatures are stated above.

For simplicity, 60C should work for all assays except CR1.



1/4

Fig. 1

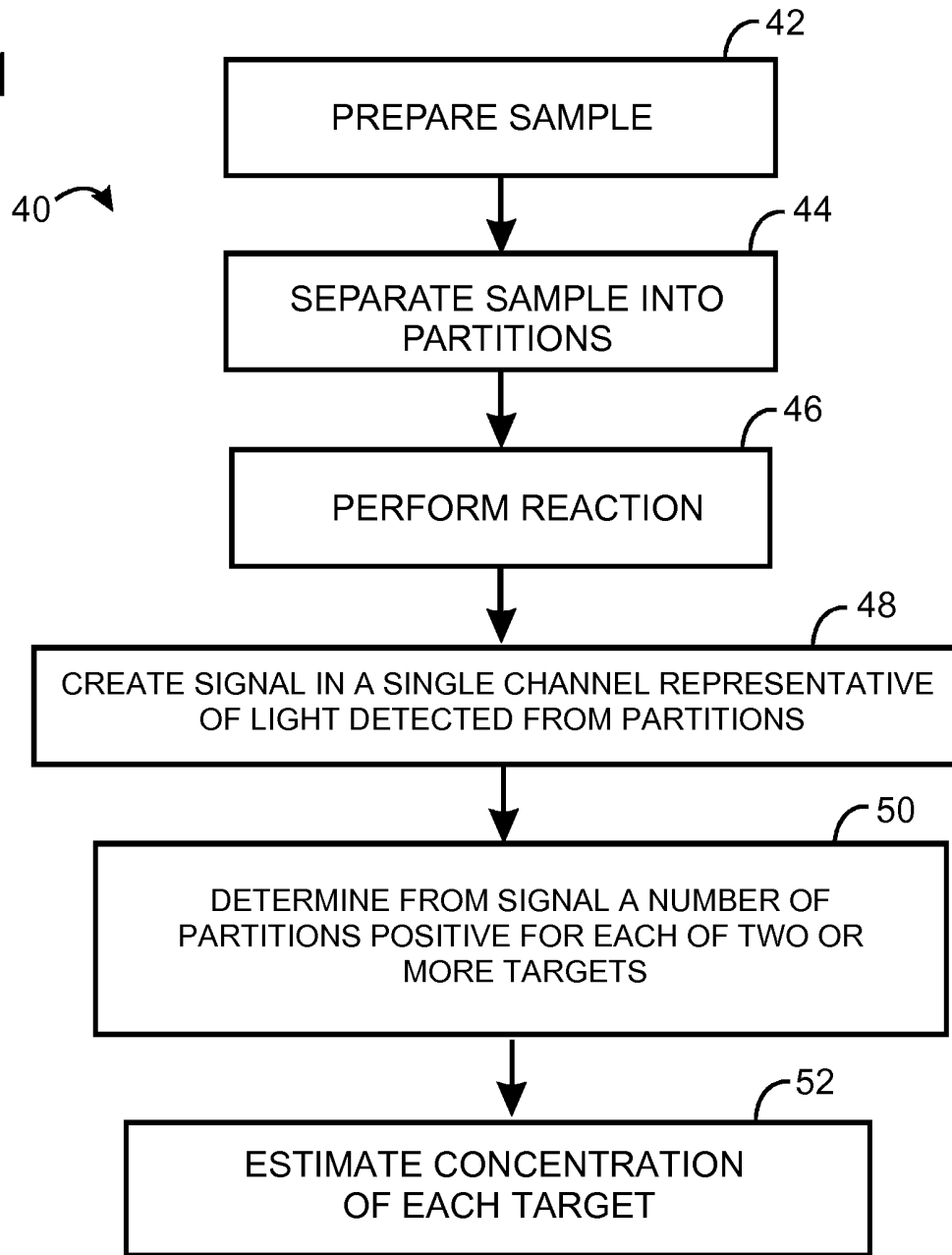


Fig. 2

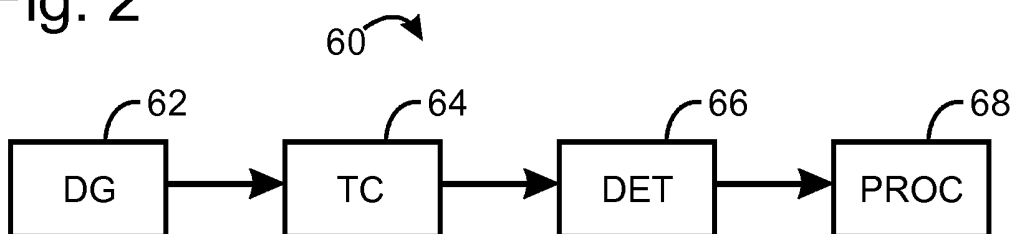


Fig. 3

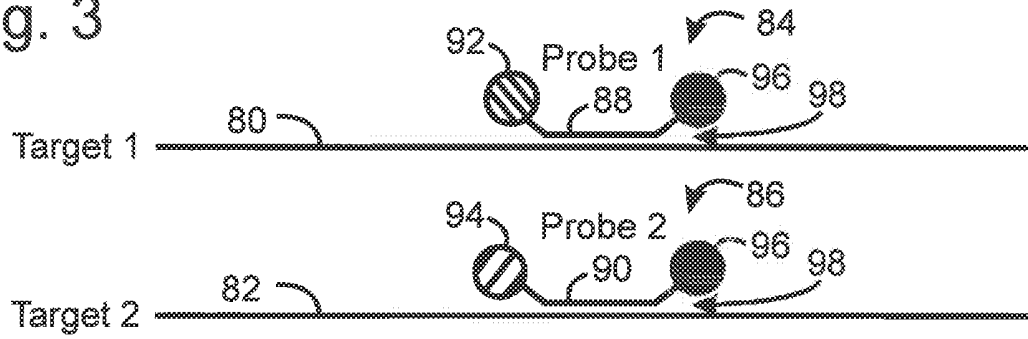


Fig. 4

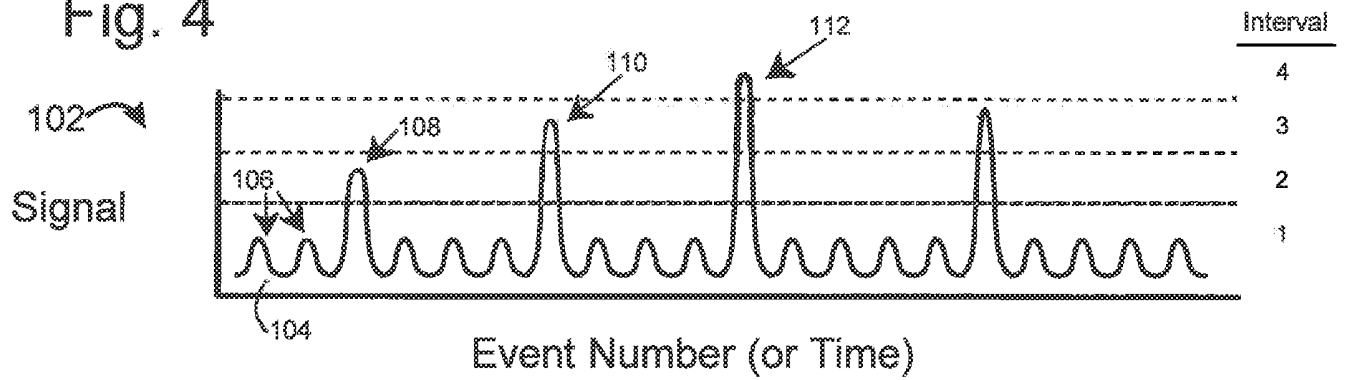


Fig. 5

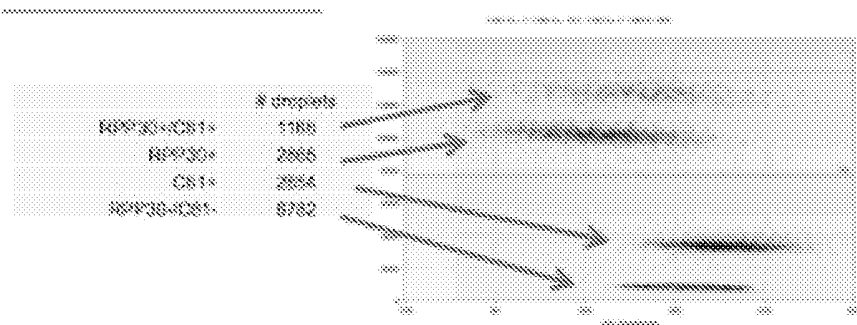


Fig. 6

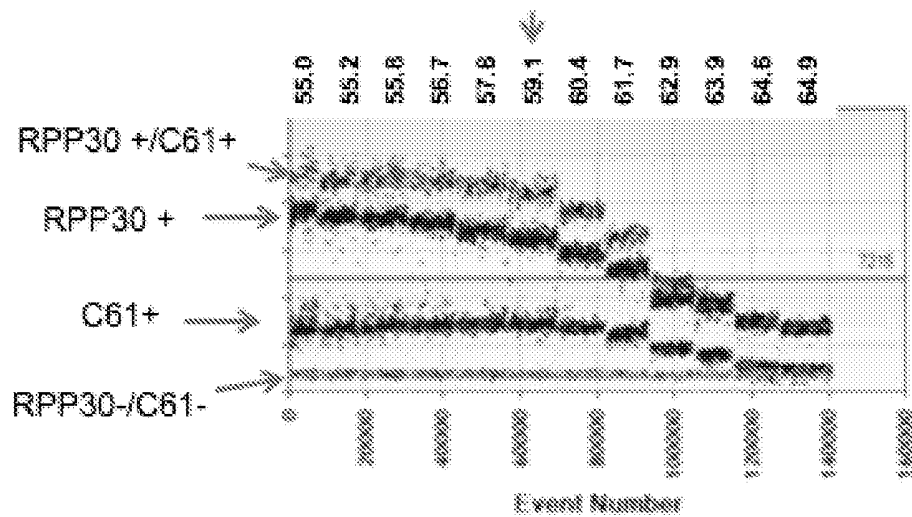


Fig. 7

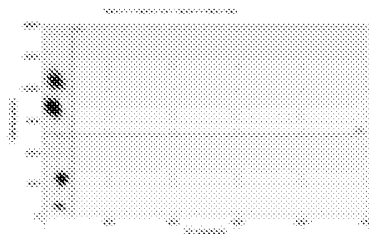
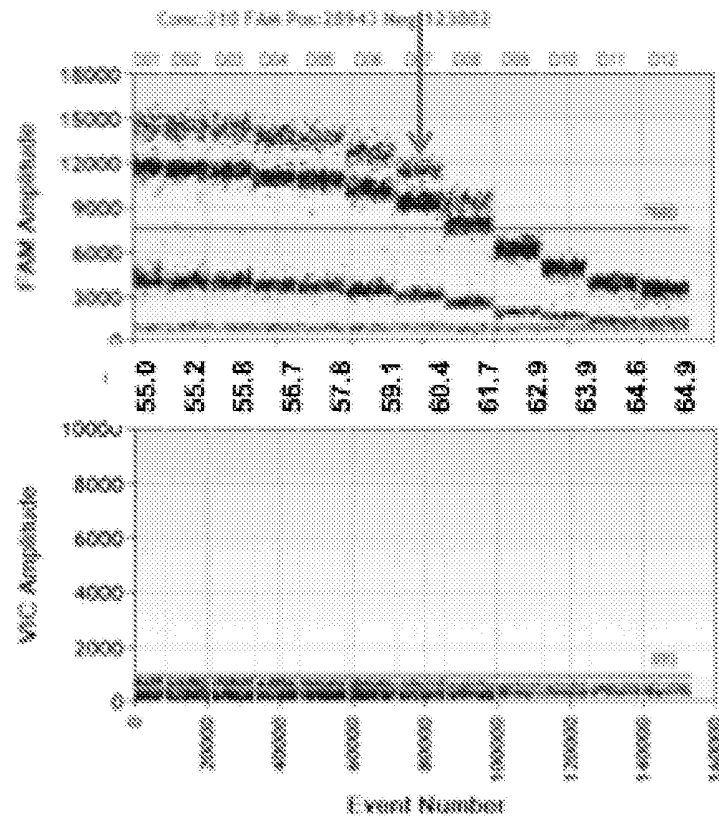


Fig. 8



Electronic Patent Application Fee Transmittal				
Application Number:				
Filing Date:				
Title of Invention:		DIGITAL ASSAYS WITH MULTIPLEXED DETECTION OF TWO OR MORE TARGETS IN THE SAME CHANNEL		
First Named Inventor/Applicant Name:		Serge Saxonov		
Filer:		James R. Abney/Margaret Burton		
Attorney Docket Number:		QLI 325P1		
Filed as Small Entity				
Provisional Filing Fees				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Provisional Application filing fee	2005	1	110	110
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Miscellaneous:				
Total in USD (\$)				110

Electronic Acknowledgement Receipt

EFS ID:	10564739
Application Number:	61510013
International Application Number:	
Confirmation Number:	6176
Title of Invention:	DIGITAL ASSAYS WITH MULTIPLEXED DETECTION OF TWO OR MORE TARGETS IN THE SAME CHANNEL
First Named Inventor/Applicant Name:	Serge Saxonov
Customer Number:	23581
Filer:	James R. Abney/Margaret Burton
Filer Authorized By:	James R. Abney
Attorney Docket Number:	QLI 325P1
Receipt Date:	20-JUL-2011
Filing Date:	
Time Stamp:	19:56:52
Application Type:	Provisional

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	\$ 110
RAM confirmation Number	7153
Deposit Account	111540
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)	

File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Provisional Cover Sheet (SB16)	20110720_Provisional_Cover_Sheet_QLI325P1.pdf	1060281	no	3
			ea980e869a838c430ef5fc0761a9573fd7b28af7		
Warnings:					
Information:					
2		20110720_Digital_Assays_w-Multiplex_Detection_PPA_as-filed_QLI325P1.pdf	186490	yes	27
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	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Specification		1	26	
	Abstract		27	27	
Warnings:					
Information:					
3	Appendix to the Specification	20110720_Appendix_Aa_QLI325P1.pdf	304785	no	10
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Warnings:					
Information:					
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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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

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

New International Application Filed with the USPTO as a Receiving Office

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