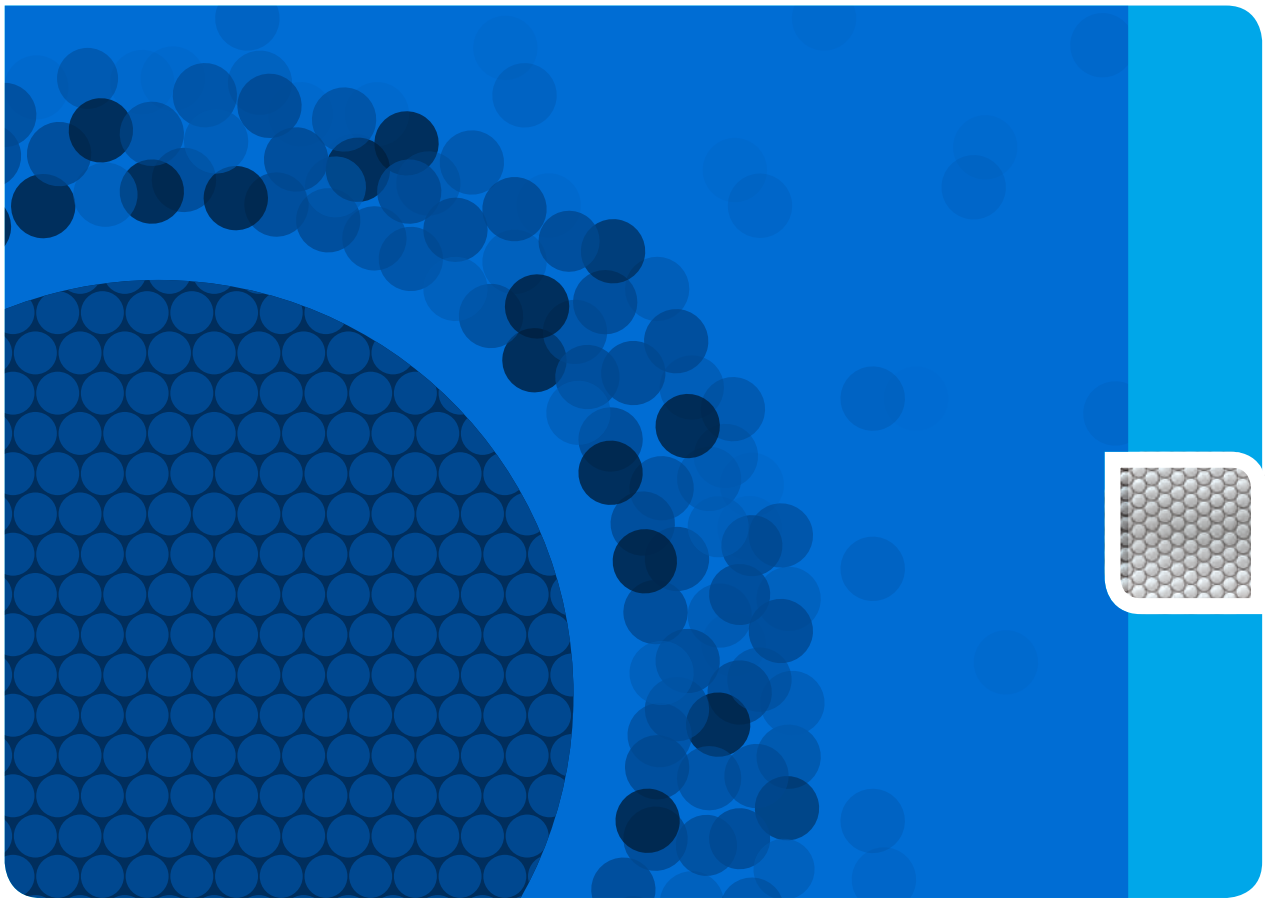

Rare Mutation Detection
Best Practices Guidelines

BIO-RAD

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1 Introduction

PrimePCR™ ddPCR™ Mutation Detection Assays

Rare mutation detection (RMD) refers to detection of a sequence variant that is present at a very low frequency in a pool of wild-type (WT) background. The challenge for RMD is the discrimination between two highly similar sequences, one of which is significantly more abundant than the other. One example of an RMD assay is detection of a low-frequency single nucleotide mutation in a cancer biopsy sample.

Bio-Rad Laboratories offers validated RMD assays for cancer mutations. Each pair of mutation and reference assays contains a single set of primers and two competitive probes, one detecting the WT allele (in HEX), and one detecting the mutant allele (in FAM) (Figure 1A). When running a mixed sample with PrimePCR ddPCR Mutation Detection Assays, the 2-D plot (Figure 1B) typically contains four clusters of droplets: (1) double-negative droplets containing no targeted DNA templates (gray circle); (2) WT-only droplets (green circle); (3) mutant-only droplets (blue circle); and (4) double-positive droplets containing both WT and mutant (MT) DNA templates (orange circle). The concentrations of the WT and MT templates are calculated based on the number of droplets in each cluster.

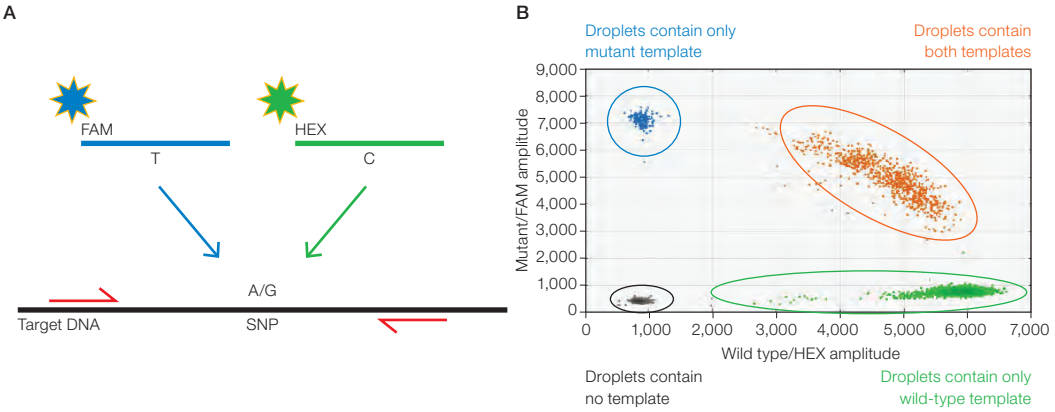

Fig.1. Mutation assay characteristics. A, schematic of a mutation detection assay with mutant allele detection in FAM and wild-type allele detection in HEX; **B**, example of the location of droplets with different templates in a 2-D scatterplot.

All validated PrimePCR ddPCR Mutation Detection Assays have been wet-lab validated in one well on mutant DNA present at 0.1% in a background of WT DNA. An even better sensitivity of 0.005–0.1% can generally be achieved by using multiple reaction wells. In addition to the wet-lab validated PrimePCR ddPCR Mutation Detection Assays, we also offer in silico validated Mutation Detection Assays for the less prevalent COSMIC (Catalogue of Somatic Mutations in Cancer) mutations. This guide focuses on Bio-Rad PrimePCR Assays, but the same principles apply to user-developed mutation detection assays.

Limit of Detection of Mutation Detection Assays

A common question when planning RMD experimental work is what the limit of detection (LOD) is. The LOD is defined as the lowest mutant concentration that can be reliably distinguished from the WT only (mutation-negative) control. The LOD is fundamentally a property not only of assay design, but also of multiple experimental factors:

1. **Assay specificity** — the ability of an assay to discriminate mutant from WT sequences is critical. If the MT probe cross-reacts with WT targets, separation between WT-only and double-positive clusters is compromised. This in turn hampers clear assignment of droplets into each of the four bins (Figure 2). Separation is also affected negatively by increasing the WT concentration. Well-designed assays can tolerate higher WT concentrations and thus screen more copies for potential better sensitivity with a fixed reaction volume. All validated PrimePCR ddPCR Mutation Detection Assays perform well with up to 130 ng of human genomic DNA (gDNA) (40,000 copies) per 20 µl reaction.

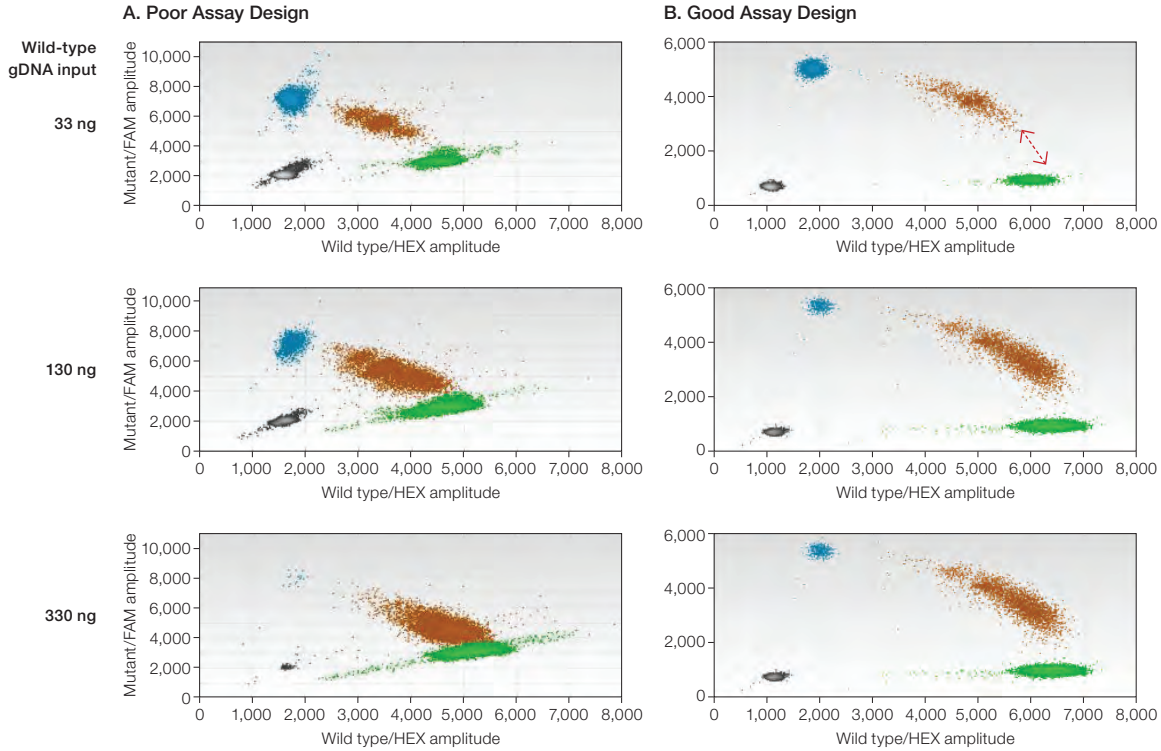

Fig. 2. Cluster separation as a function of assay design and DNA amount. Examples of cluster separation (red arrow) as a function of DNA load amount when using a poorly designed assay (**A**) vs. a well-designed assay (**B**). As increasing amounts of DNA are added, separation between double-positive and wild-type clusters decreases.

2. **Sample availability and quality** — in most cases, sample amount and quality are the limiting factors for achieving low LOD. For example, if 3,000 amplifiable genome equivalents are extracted from 1 ml of blood, it is impossible to achieve better than a 1 in 3,000 LOD on this sample (Appendix B: Converting Nanograms to Copies for Human DNA). In addition, different DNA preparations and sample types can limit the number of amplifiable copies available. Cell line DNA is relatively intact and gives the best results for separation, efficiency, and cluster density.

   Damaged samples like formalin-fixed, paraffin-embedded (FFPE) tissue, bisulfite-converted gDNA (BSC), and cell-free DNA (cfDNA) generally yield highly damaged DNA which can alter cluster amplitude and appearance and affect separation, therefore potentially affecting LOD (Figure 3). In addition, formalin fixation causes artifactual C>T transitions that can adversely impact LOD.

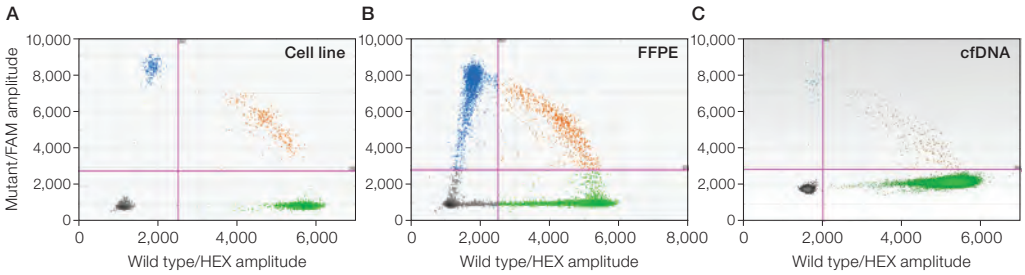

Fig. 3. Changes in cluster separation due to quality and type of DNA sample. Using the same mutation detection assay, cluster separation differs greatly when using cell line DNA (**A**), FFPE sample (**B**), or cell-free DNA (**C**).

1. **Inhibitors** — strong PCR inhibitors, such as detergents used in lysis buffers, can alter the location of droplet clusters, causing a loss of sensitivity. A vast excess of degraded DNA is another source of PCR inhibition and reduced sensitivity. FFPE samples, for example, can contain PCR inhibitors and are highly degraded; often <10% of the sample (as measured by UV spec or DNA dye binding methods) is amplifiable. As shown in Figure 4, dilution or reduction in sample loading usually solves this problem.

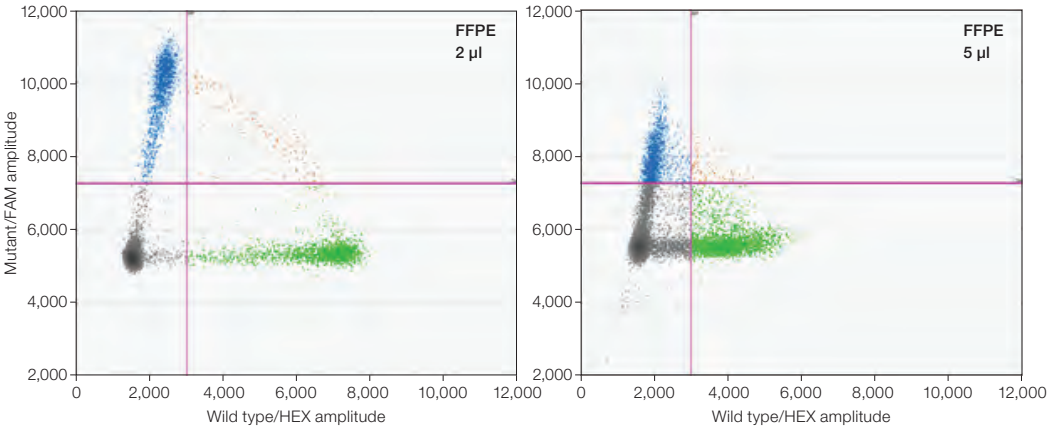

Fig. 4. Inhibition causes loss of cluster separation. This sample contains inhibitors that can cause loss of fluorescence amplitude, thereby affecting cluster separation and quantification. Cluster separation improves upon dilution (5 µl vs. 2 µl).

1. **False-positive droplets** — there are two types of false-positive droplets: those seen in the no template controls (NTCs), indicating contamination, and those seen in the WT only (mutation-negative) controls (Figure 5). False-positive droplets negatively impact the overall LOD and the false-positive rate is a critical factor in achieving a very low LOD and accurate quantification at low MT concentration. The causes of false-positive droplets, discussed in more detail below, include contamination in the laboratory or of reagents, poor droplet handling, poor sample quality, polymerase-induced errors, template sequence, and instrument artifacts.

   a. Sample handling: one of the main sources of false-positive signal is contamination of the reagents and work space. These false positives will be most visible in your NTCs, although they will also be present in your samples (Figure 5A). Another source of false-positive signal seen in the NTCs are non-uniform droplets caused by poor droplet handling (Figure 5B).

   b. Type and quality of the sample: DNA sequence and damage can cause an increase in false-positive droplets in the WT-only (mutation-negative) control because they lead to polymerase-induced errors (Figure 5C). For example, formalin fixation causes C>T transitions by deamination of cytosine. Uracil DNA glycosylase (UDG or UNG) treatment of FFPE samples can prevent false-positive signal introduced by formalin fixation. For this reason, we recommend adding UDG to FFPE samples being analyzed for RMD.

      To estimate the false-positive rate, a minimum of three negative controls that contain only WT DNA at a concentration, or concentrations, relevant to tested samples is recommended (for example, if you are using one well per sample at around 130 ng input sample, there should be three negative control wells with WT-only DNA at 130 ng per well). The WT DNA control should ideally be from a similar source and extracted with the same sample preparation method as tested samples (for example, if testing FFPE samples, the WT control should ideally be an FFPE sample as well).

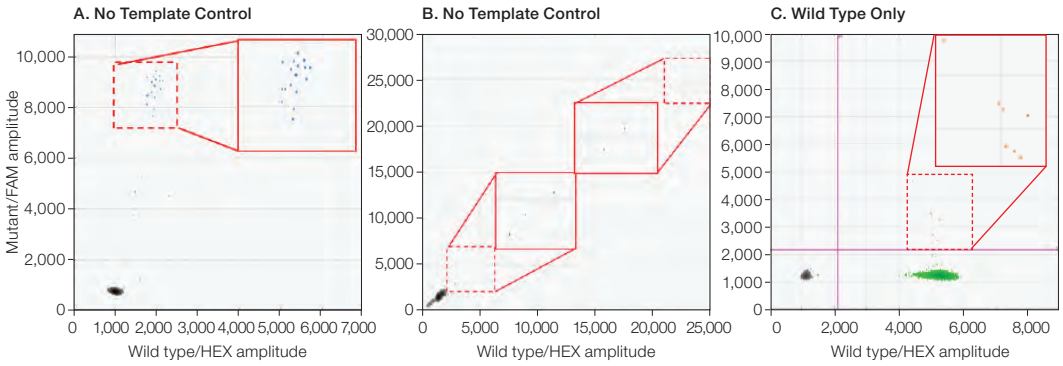

Fig. 5. False positives in no template controls (NTCs) and wild-type–only (mutation-negative) controls. A, evidence of contamination in the NTC; **B**, evidence of mishandled droplets, both shredded (lower box) and coalesced (upper box) which lie on the 45° axis; **C**, false-positive droplets in wild-type–only (mutation-negative) controls.

1. **Error** — LOD is affected by intrinsic error, such as poor assay design, extrinsic error, such as poor sample handling, and stochastic error. Error in mutation detection experiments is influenced by the factors discussed above and by additional sources like subsampling error and Poisson error. Poisson error is best used for technical replicates, while total error best approximates human and biological error. For a more in depth discussion of error, see the Droplet Digital™ PCR Applications Guide, bulletin 6407, Appendix B.

Limit of Detection and the Rule of Three

A key contributor to LOD in ddPCR is the amount of sample screened. One convenient rule of thumb for estimating theoretical LOD for a given amount of sample loaded is the Rule of Three (see box). For example, if 10 ng of amplifiable human gDNA (~3,000 copies) is assayed in a single reaction well, then three positive events out of 3,000 screened (0.1% sensitivity) is the theoretical LOD for this reaction. Table 1 depicts the amount of starting material required to achieve a certain LOD based on the Rule of Three. See Appendix B for a theoretical estimate of reaction LOD based on sample input, but remember that the real-world assay and experimental factors described above will hamper achievement of the theoretical LOD.

LOD and the Rule of Three

According to Poisson's law of small numbers, if there is a random distribution of quantifiable, independent events, predictions can be made about the likelihood with which those events occur. Based on the number of molecules and partitions, Poisson predicts the degree of spread (95% confidence interval) around a known average rate of occurrences. In order to reach 95% confidence that a sample frequency is 1 in 1,000, you need to identify 3 in 3,000 events, hence the Rule of Three.

Table 1. Minimum starting material requirements for achieving a low limit of detection.*

	Required Starting Material (human)			Number	Number
LOD	Total Copies	Diploid Cells	Amount of DNA	of Droplets	of Wells
1 in 1,000	3,000	1,500	0.010 µg	1,500	1
1 in 10,000	30,000	15,000	0.10 µg	15,000	1
1 in 100,000	300,000	150,000	1.0 µg	150,000	8

* This assumes a copy number load of 2,000 copies per µl (cpm) per well and 15,000 droplets per well.

2 Experimental Setup

Setting Up a Mutation Detection Reaction

An optimal mutation detection experimental design requires the following elements (Table 2), discussed in detail below.

Table 2: Strategy for optimal experimental design.

Component	Purpose
No template control (NTC) wells	Identify environmental contamination
Wild-type–only (mutation-negative) control wells	Estimate assay/sample false-positive rate
Mutation-positive control wells	Verify assay performance and identify expected cluster positions to inform thresholding
Experimental (sample) wells	Experiment

Important tip: No template controls monitor good laboratory practice.

Good Laboratory Practices for Mutation Detection

When performing mutation detection experiments, it is essential to have a clean working environment. Wipe down working surfaces (bench, chair, tips, pipets, etc.) with bleach every time. Separate working areas for assay setup (cleanroom or amplicon-free area) and template addition (PCR hood) are recommended. The droplet reader should ideally be placed in a room separate from reaction setup. Changing gloves often and not reusing cartridges, gaskets, pipets, or trays also limits contamination.

Important tip: Do not dilute or store high copy (synthetic template) positive controls in reaction preparation areas.

Testing Clean Technique

After setup of your Droplet Digital™ PCR (ddPCR™) area, it is good practice to run several no template control (NTC) wells to verify clean technique. As digital PCR is extremely sensitive, it is not unusual to find that previously "clean" environments lead to positives in the NTCs. It may require several rounds of cleaning laboratory space and equipment to eliminate NTC false positives. After initial tests are run, it is still imperative to run a few NTCs with every plate.

Wild-Type–Only (Mutation-Negative) Wells

Wild-type (WT)–only (mutation-negative) wells are critical for estimating the false-positive rate due to sample type or assay performance. Running several WT-only wells improves false-positive estimation (see below). As false-positive rate is in part a function of the amount of sample loaded, WT-only wells should ideally be run at a concentration similar to that expected for the samples (for instance, if 100 ng of sample will be tested, run 100 ng WT-only sample in a control well). This WT-only material should ideally be from a sample preparation analogous to samples tested (for example, formalin-fixed, paraffin-embedded [FFPE] samples).

Important tip: If treating FFPE samples with uracil DNA glycosylase (UDG) prior to ddPCR (see below and Appendix A), make sure to also treat any FFPE WT controls in the same manner.

Mutation-Positive Control Wells

Positive controls are critical for confirming assay performance and useful for predicting positive cluster positions. We recommend using the same sample type as used in the experimental wells, when possible. Good options for positive controls are:

1. **Cell line DNA (for example, Horizon Diagnostics or ATCC)**
2. **Plasmid controls (multiple vendors)** — these should be fully linearized. Use a 500 bp genomic DNA fragment with the mutation of interest at the center. To ensure complete digestion, put several copies of a restriction digest site that is compatible with the mutation detection assays on either side of the insert. Undigested plasmid is not a good template for ddPCR — it will produce extremely rainy results (Figure 6).
3. **Gene fragments (for example, IDT or Operon)** — these are double-stranded, sequence-verified blocks of DNA.

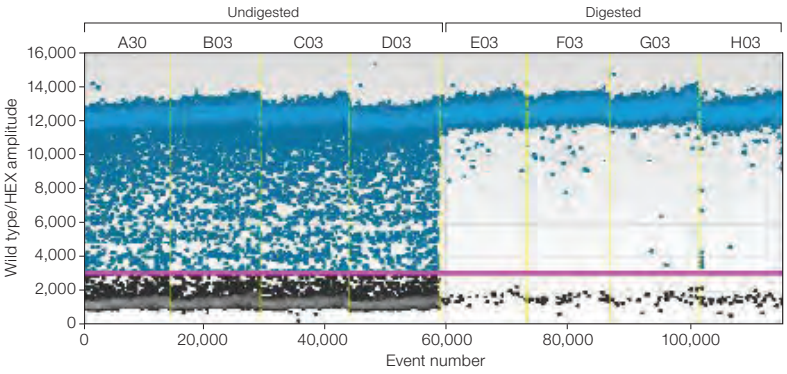

Fig. 6. Intermediate droplets (rain) caused by an undigested plasmid sample. Restriction digest permits full template access and significantly improves quantification.

Note that HPLC-purified long synthetic oligos (for example, ultramers) are not good positive controls for ddPCR. Standard oligo synthesis produces a mixture of DNA fragments of different lengths. These non–full-length fragments appear in ddPCR data as rain — droplets that fall between the positive and negative clusters.

Synthetic positive control templates like plasmids or gene fragments are provided at high concentrations, and if not handled properly can readily contaminate reactions or work areas. When diluting these materials to get within the dynamic range of the system, work in a post-PCR area and take care not to bring the high-concentration stocks into the pre-PCR labs. Changing gloves often and wiping all surfaces with bleach is also essential. It is very easy to get contamination from high-concentration stocks.

Note that the vendor-provided quantification of the synthetic template may not be accurate. See Appendix C for a table on converting molar concentration to copies to assist in estimating expected results.

Also note that DNA stored at low concentration will stick to plastic tubes, so the concentration may change over time. Because synthetic template contains only the fragment of interest, a 20,000 copy per µl stock has a very low total DNA concentration. For long-term storage of templates at low concentrations, dilute them in a buffer with a carrier such as polyA DNA. We recommend 100 ng/µl polyA in TElo buffer.

Experimental (Sample) Wells

Each ddPCR reaction contains the following components (see Appendix A for reaction setup details):

- Master mix: 2x ddPCR Supermix for Probes (No dUTP)
- Wild-type assay
- Mutant assay
- Restriction enzyme (recommended)

In order to achieve optimal results, the input DNA template amount needs to be adjusted for the following reasons:

1. **Instrument dynamic range** — QX100™ and QX200™ Droplet Digital PCR Systems allow accurate quantification from 0.5 to 5,000 copies of target per µl (100,000 human genome equivalents per reaction, or 330 ng of intact human genomic DNA). For synthetic templates, be careful to stay within the dynamic range of the system. In most cases, clinical samples are within the dynamic range of one well, detecting 1–100,000 copies per well; when concentrations of your sample are low, multiple wells can be merged to ensure accurate quantification of rare mutations.

2. **Assay performance** — due to challenges in cluster separation, not all assays perform well under maximal (5,000 copies/µl) loading conditions. PrimePCR™ ddPCR™ Mutation Detection Assays are validated using a loading concentration of around 2,000 copies per µl (130 ng genomic DNA) and many of them perform well at concentrations up to 5,000 copies per µl (330 ng genomic DNA). If using your own assays, we recommend running them on a dilution series of the DNA template in order to estimate the appropriate operating range of the assays (Figure 2).

Restriction Digestion

DNA fragmentation by restriction enzyme digestion enables optimal accuracy by separating tandem gene copies, reducing sample viscosity, and improving template accessibility. All Bio-Rad PrimePCR Assays are validated with restriction enzyme digestion, and restriction digest is recommended for optimal performance. Check the PrimePCR website (www.bio-rad.com/primepcr) for recommended restriction enzymes. For convenience, all restriction enzymes recommended for use with PrimePCR Assays are compatible with the ddPCR Supermix and can be added directly into the reaction without additional incubation time (see Appendix A).

For fragmented or low-concentration DNA, assays may work without restriction enzyme digest. However, digestion can still increase the precision, reproducibility, and accuracy of ddPCR. Some targets (for example, *HRAS G12V*) have inhibitory secondary structures that negatively impact assay performance in the absence of digestion. In addition, if you have a wide range of input samples, digestion can help standardize your results. For example, cell-free DNA purified from plasma can have highly variable lengths and amounts of DNA. In the majority of samples, digestion would not be necessary. However, some samples can have too much large-fragment DNA, which can compromise your relative mutant counts in longitudinal studies. Random shearing of DNA helps in most cases, although it may lead to less sensitivity due to destruction of mutant templates and an increase of rain in the assay.

UDG Treatment of FFPE Clinical Samples

One type of damage caused by formalin fixation (for example, FFPE samples) is the deamination of C to U (Do and Dobrovic 2012). This damage can result in C>T transitions following PCR amplification, directly increasing the false-positive rate. A simple but effective strategy for reducing spurious C>T transitions is pretreatment of FFPE samples using uracil DNA glycosylase (UDG) (Figure 7). This can be done directly during ddPCR reaction setup, or prior to ddPCR (see Appendix A for details). If FFPE samples are used as WT-only controls, treat these materials in the same manner as the experimental samples.

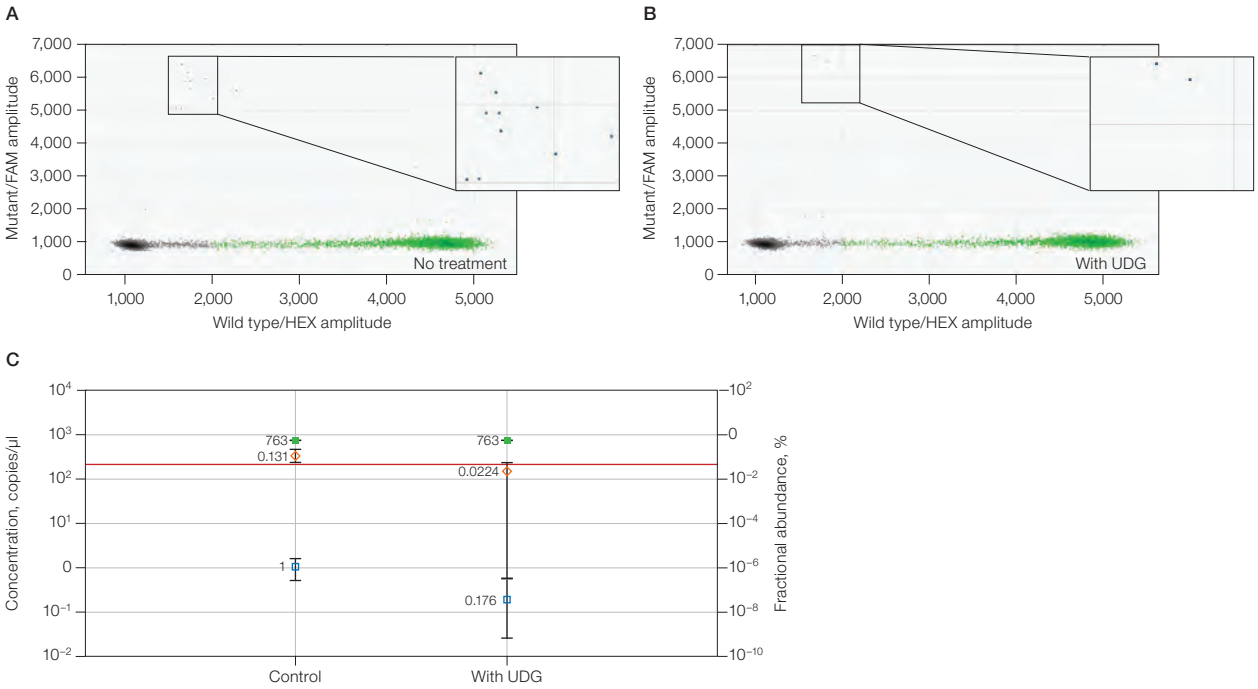

Fig. 7. Pretreatment of formalin-fixed, paraffin-embedded (FFPE) sample with uracil DNA glycosylase (UDG) prevents false-positive signal. Treatment of DNA with formalin causes multiple forms of DNA damage. Treatment with UDG digests deaminated cytosines, removing cytosine>thymine (C>T) false positives. When pretreated with UDG, an FFPE sample (M2W, grossly normal) showed significantly lower *G13D* mutation-positive droplets (**A** and **B**, events 12 vs. 2, respectively), altering the sample call from mutant (MT) to wild type (WT). The fractional abundance of *G13D* mutants in the control (no treatment) compared to the sample with UDG is significantly different, indicating that the UDG treatment may result in a different call (**C**). WT template concentration (■); mutant template concentration (■); MT fractional abundance (◆).

Reference

Do H and Dobrovic A (2012). Dramatic reduction of sequence artefacts from DNA isolated from formalin-fixed cancer biopsies by treatment with uracil-DNA glycosylase. Oncotarget 3, 546–558.

3 First Experiments

PrimePCR™ ddPCR™ Mutation Detection Assays are all fully validated using a positive control spiked into a background of wild-type (WT) genomic DNA. Nonetheless, for the new user, we recommend using the experiments described here for each new assay to familiarize yourself with the workflow, environment, and samples. Below is a suggested format for testing the false-positive rate (FPR) and limit of detection (LOD) of your system, prior to running your samples.

Assessing Good Technique with No Template Controls (NTCs)

Droplet Digital™ PCR (ddPCR) requires careful handling of reagents and droplets. Due to the sensitivity of the ddPCR platform, extremely low levels of contamination can be detected. When examining rare mutations, it becomes critical to monitor contamination. One of the first experiments a new user should perform is a test of a few PCR plate columns of NTCs using your assay. This will test the presence of contamination in your laboratory, as well as set a baseline for future experiments.

Estimation of the False-Positive Rate (FPR)

When measuring very rare events, such as false-positive droplets in mutation detection experiments, more measurements enable a more accurate estimation. In the experiment below (Figure 8A), false-positive events were measured using the same WT DNA sample in eight independent wells (Figure 8A, columns 2–9). In these individual wells, 0, 0, 1, 2, 3, 3, 3, and 0 mutation-positive droplets were observed. The merged metawell (Figure 8A, column 1) shows 12 positives total, indicating an average of 1.5 events per well, with tighter error bars than all other individual wells (Figure 8B). If false positives were measured using only one well, we would easily overestimate or underestimate the FPR.

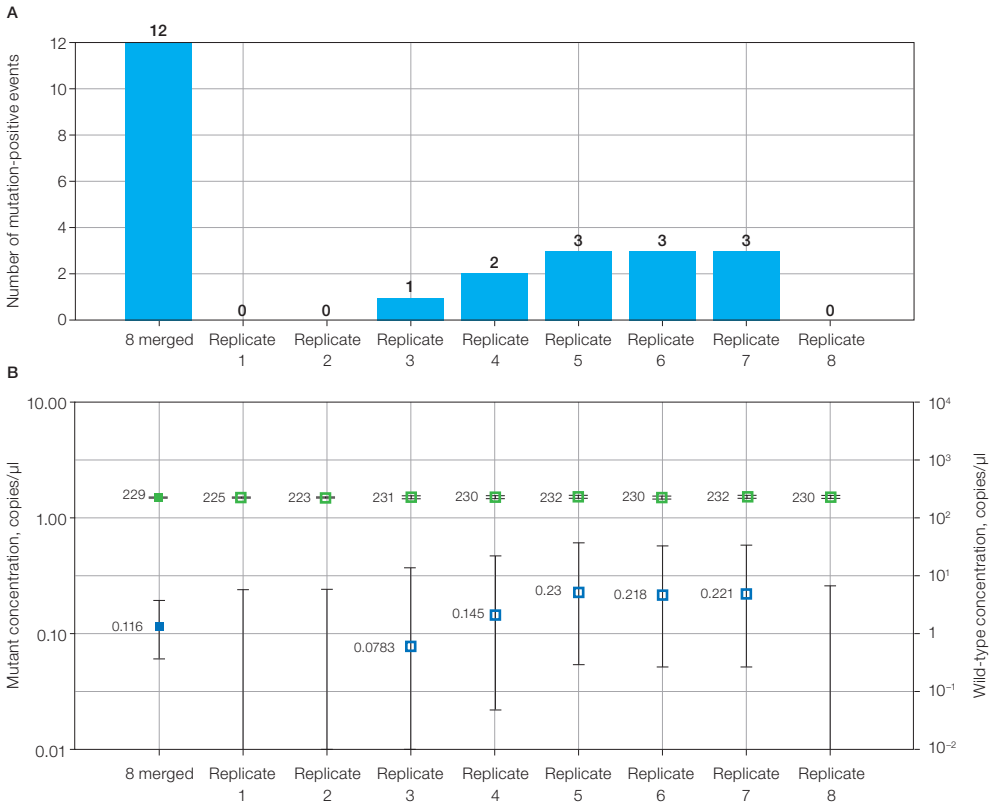

Fig. 8. Estimating false-positive rate (FPR) and the benefit of merged wells. A, the number of mutation-positive events seen in the wild-type (WT)–only wells, both merged (column 1) and independently (columns 2–9); **B**, concentrations and error bars associated with merged and independent wells. Error bars for merged events are lower than for individual wells. WT template concentration (■); mutant template concentration (■).

Empirical Determination of Limit of Detection

A strategy for empirical determination of limit of detection (LOD) is presented in Table 3. Run a plate containing NTC wells, WT-only (mutation-negative) control wells, and a serial dilution of positive control mutant (MT) template in a constant background of WT DNA. Figure 9 shows example data using a single well per sample and 130 ng of input DNA per well. For greater sensitivity, this setup can be modified to use multiple wells per dilution depending on amount of input DNA and sensitivity desired. See Appendix B for required amount of sample needed to achieve desired sensitivities and Appendix D for a sample plate layout.

Table 3. Strategy for empirical determination of LOD in a single well.

Number of Wells	Purpose	DNA Template Content
1 NTC	Monitor contamination	No DNA
2–3 wild-type–only (mutation-negative) control	Monitor false positives	Wild-type–only genomic DNA at 130 ng per reaction
4–5 serial dilutions of mutation-positive control	Positive controls for thresholding and making LOD call	Serial dilution of mutant spiked into 130 ng/reaction of wild-type genomic DNA. Plan the serial dilution to encompass the desired LOD

A reasonable estimate of the limit of detection is the dilution that shows a statistically significant difference from the negative controls; this would be the lowest MT concentration detected in a mixed template sample where the lower error bar of the measured MT concentration does not overlap with the upper error bar of the measured MT concentration in the WT-only (mutation-negative) control (Figure 9, dotted red line).

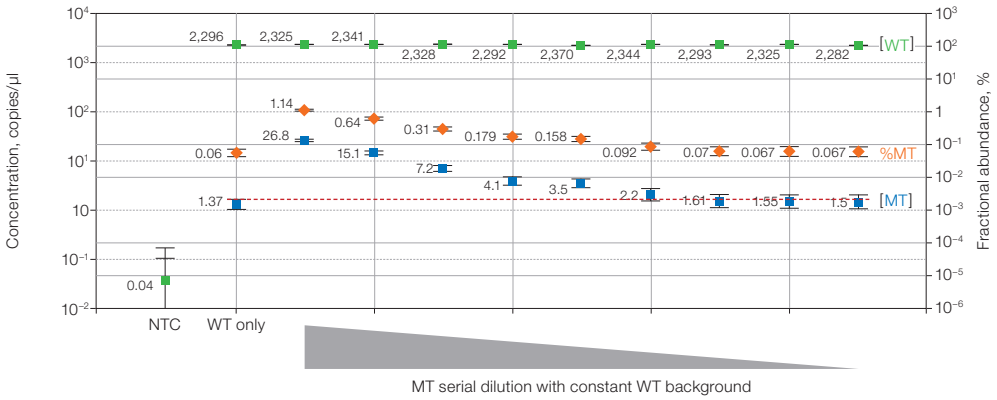

Fig. 9. LOD validation for rare mutation detection. Wild-type (WT) template concentration in copies per µl (cpm) (■), mutant template cpm (■), and fractional abundance of mutant to WT template (◆). The upper limit of the mutant concentration error bars in the WT-only (mutation-negative) control does not cross the lower limit of the mutant concentration error bars in the serial dilution (dotted red line). This indicates a statistically significant difference between the negative control and the mutant dilutions and allows us to conclude that even the lowest concentration of mutant template tested is still within the assay's LOD.

Testing Unknown Samples

It is essential to include NTCs, WT-only (mutation-negative) controls, and MT-positive controls when testing any unknown sample (Table 4). If the total DNA amount of the unknown samples varies significantly, multiple WT-only controls are recommended at a variety of DNA concentrations to best estimate the FPR at different load amounts. (See Appendix E for an example of how FPR changes with DNA concentration.)

Table 4. Recommended minimal experimental setup, assuming no replicates.

Number of Wells	Purpose	DNA Template Content
1 NTC	Monitor contamination	No DNA
>1 wild-type–only (mutation-negative) control	Monitor false positives	Wild-type–only genomic DNA at 130 ng per reaction.*
1 mutation-positive control	Positive control for thresholding	Mixed template with wild-type genomic DNA at 130 ng and mutant genomic DNA above 2.6 ng (>2%) per reaction
1 per sample	Measure concentration	Unknown sample DNA

* When using low-concentration partially degraded samples, for example formalin-fixed, paraffin-embedded (FFPE) or cell-free DNA (cfDNA) clinical samples, an input of 130 ng amplifiable material per negative control well is not possible. In these cases, loading negative control wells with the maximum amount of amplifiable material expected in unknown samples is recommended.

For maximum sensitivity detection, it may be necessary to use more than one well per sample. Again, depending on the amount of sample available, you may have to merge multiple wells to achieve 1 in 10,000 detection. See the troubleshooting section for examples.

4 Data Analysis

Evaluation of Data Quality

It is good practice to systematically evaluate your droplet data. QuantaSoft™ Software attempts to automatically threshold the data, but for rare mutation detection it is often optimal for the operator to manually set the thresholds.

1. **Examine data after droplet reading, making note of data quality.**

   a. Evaluate wells for overall accepted events (if below 10,000 events, you should carefully evaluate the data for additional problems as noted below).

   b. Check wells for aberrant fluorescence amplitudes. Significant amplitude differences and concentration differences between replicate samples (Figure 10) indicate poor handling or mixing of samples.

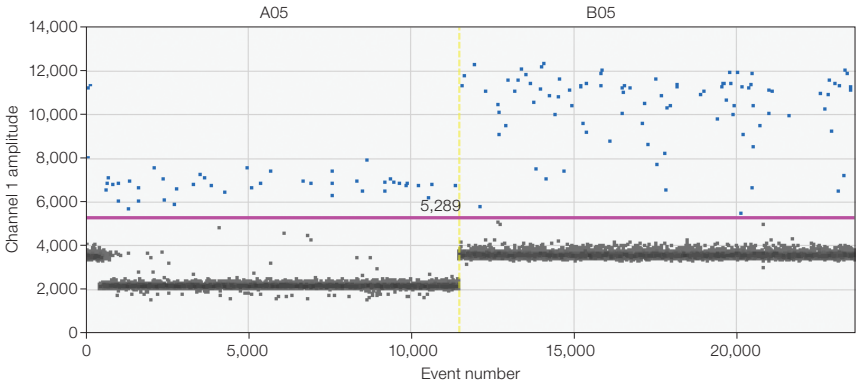

Fig. 10. Differences in fluorescence amplitudes can indicate a data quality issue. The above wells are technical replicates with template-positive droplets (■) and template-negative droplets (■). Poor droplet generation due to poor mixing, poor handling, or precipitates in the sample can cause the observed differences in fluorescence amplitude and droplet size.

c. Make note of wells with spray patterns on a 45° axis, which is indicative of poor droplet quality or problem samples. If data appear as a 45° line and there are only a few droplets (Figure 11A) then mark those droplets as negatives using the threshold tools. If the spray pattern is excessive (Figure 11B), then disregard the well.

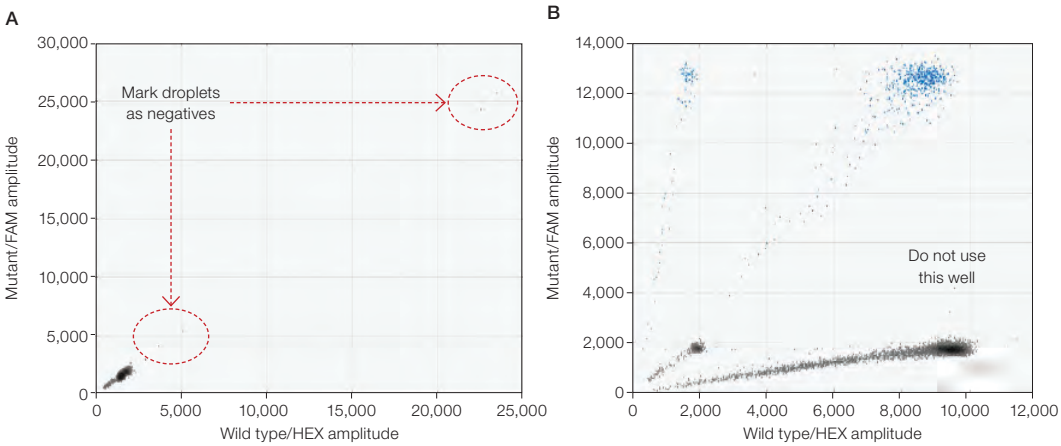

Fig. 11. Identifying droplet quality issues. A, shredded and coalesced droplets (dotted circles) will lie on a 45° axis and when present at low levels can be thresholded out using QuantaSoft Software tools; **B**, occasionally, excessive shredding wil occur and a well will need to be discarded.

1. Examine and threshold control wells using both 2-D and 1-D plots.

a. As a first pass, the three types of control wells (no template controls, wild-type [WT]–only [mutation-negative], and mutation [MT]-positive) should be examined; thresholding can then be further adjusted as needed (b-g below). Select all control wells and examine cluster quality by 2-D plot. For proper thresholding, clear separation between clusters should be apparent. The MT DNA concentration in the MT-positive control well should be high enough to correctly locate the edges of the single-positive (WT) cluster and the double positives (WT and MT). Using the same threshold for all controls and samples ensures that the same false-positive rate applies to all reactions. This can be done with any of the thresholding tools in QuantaSoft Software (Figure 12).

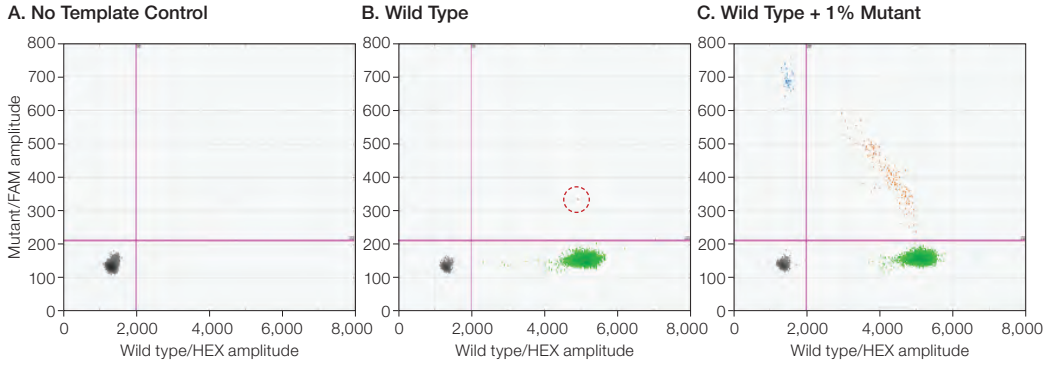

Fig. 12. 2-D view of control wells. A, no template control well; **B**, wild-type–only (mutation-negative) well with a single double-positive false-positive droplet (circle); **C**, 1% mutation-positive control well. Identical thresholding was applied to all wells taking into consideration location and separation of clusters and avoidance of low amplitude fluorescent droplets.

b. NTC wells: examine all NTC wells, adjusting the threshold as needed. Drawing thresholds above random low fluorescence amplitude droplets is acceptable, as long as you are not cutting off too many true positives from your WT and MT controls (generally one to five droplets) (Figure 12A). If positives are at high fluorescence amplitude in either the WT or MT channels, contamination is a problem. If more than two NTC wells have more than five to ten positive droplets total, the data collected using this plate are questionable. Go back and decontaminate bench and pipets, get fresh water, master mix, assay, samples, and begin again. Double-positive droplets (Figure 5B) in the NTC well are more likely to be carryover or aberrant droplets (double-positive without two larger single-positive populations are highly unlikely).

c. WT wells: examine the WT-only wells to determine the false-positive rate (Figure 12B). The WT DNA concentration should be no less than the DNA input of the unknown samples in order to correctly estimate the false-positive rate. If you have unknowns with a wide distribution of template amounts, then consider adding more WT-only controls at different input template amounts and threshold samples using the appropriate WT control.

d. MT wells: The expected position of true MT positives can be estimated using the MT-only control well. Setting the thresholds between the four clusters in the MT wells and examining the WT well will reveal which droplets in the WT well are false positives. (Figure 12B red circle and 12C). After this thresholding step, the Ratio tab should be used to ensure that the measured fractional abundance of mutants is the expected amount (for example, 1%).

e. Select all samples and controls and redraw the thresholds based on the control thresholds settings.

f. Check each set of replicate wells for reproducibility of both fluorescence amplitudes and concentration calls. At extremely low concentrations this variability will increase according to normal error (Figure 13).

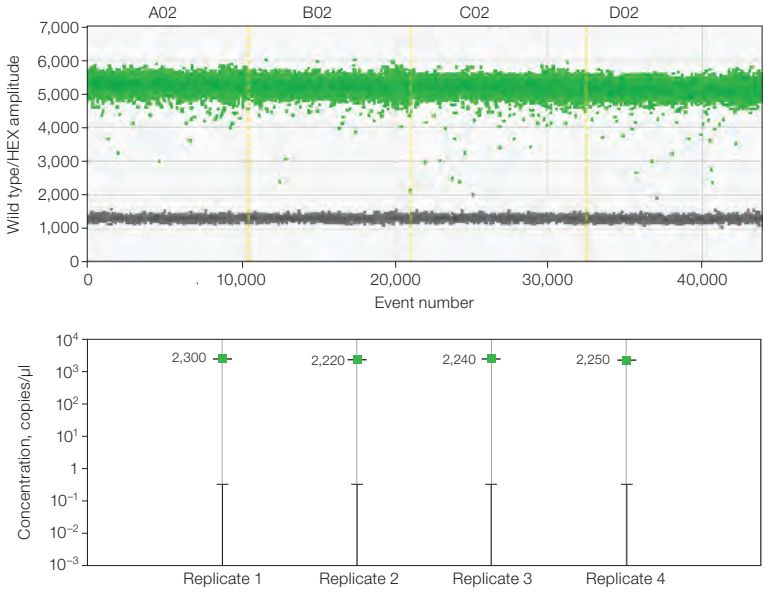

Fig. 13. 1-D plot of fluorescence amplitudes in replicate wells. A, four replicate wells (as indicated by dotted yellow line) with identical fluorescence amplitudes; **B**, four replicate wells with identical concentration calls, which can now be merged.

g. Merge wells and examine data using concentration and fractional abundance graphs (Figure 14).

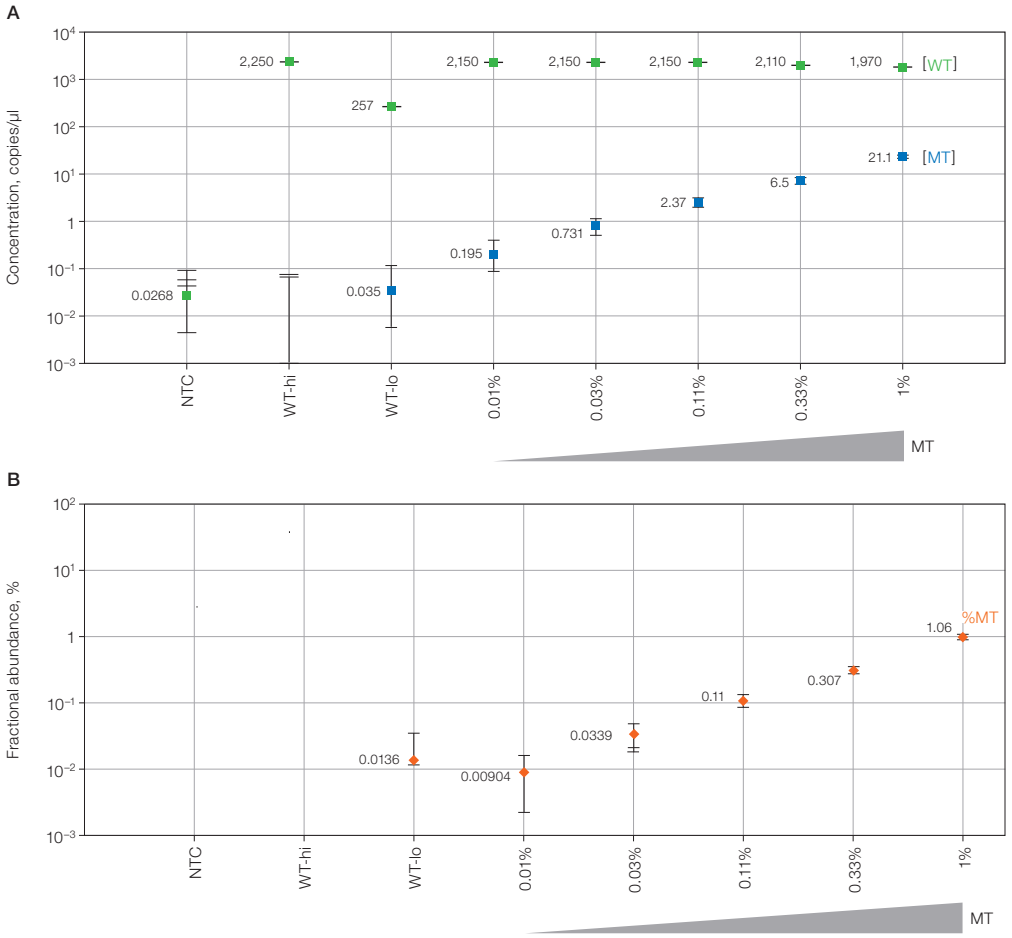

Fig. 14. Merged well concentration and fractional abundance. A, concentration calls for wild type (WT, ■) and mutant (MT, ■) including 95% confidence interval error bars; **B**, fractional abundance of [MT] in [WT+MT] background (◆) in a threefold dilution series. WT-hi and WT-lo are control template concentrations that flank the expected range of WT template concentration in the tested sample.

QuantaSoft Software has flexible visualization and analysis tools for thresholding data. A simple and efficient thresholding strategy is described here (Figure 15):

- Select all the wells containing a given assay. The 2-D plot (Figure 15A) provides information on cluster location and helps to identify the edges of each cluster for later 1-D thresholding. Use the tool on the left panel to select a rough threshold
- Switch to the 1-D view (Figure 15B) and use the multiwell threshold tool to adjust the channel 1 threshold to sit just below the positive cluster in the MT-positive control wells. It is sometimes easier to estimate the double-positive cluster edge with 1-D view and thus avoid overestimating the false positives. Confirm the correct thresholding by looking back into the 2-D view

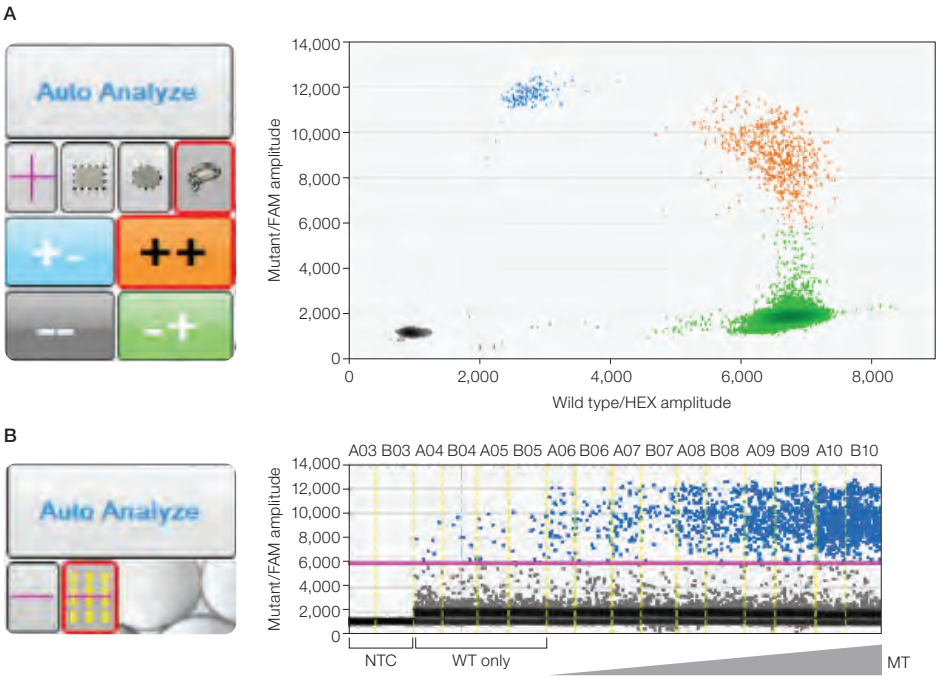

Fig. 15. Threshold tools in 2-D and 1-D plots. A, thresholding tools used to assign droplets to clusters in 2-D plot; **B**, thresholding tools for multiwell thresholding in 1-D plots. A03–B03, NTCs; A04–B05, wild-type control wells; A06–B10, mutation-positive control wells.

Confidently Calling a Positive Sample

The quick and short way to call a sample positive is by examining non-overlapping error bars (95% CI) between the WT-only (mutation-negative) control and the test samples (Figure 16). A *t* test can be used to determine if negatives and positives are significantly different. If the 95% CI error bars are non-overlapping, then a *t* test will always show significance. If the error bars overlap, then the *t* test sometimes shows significance.

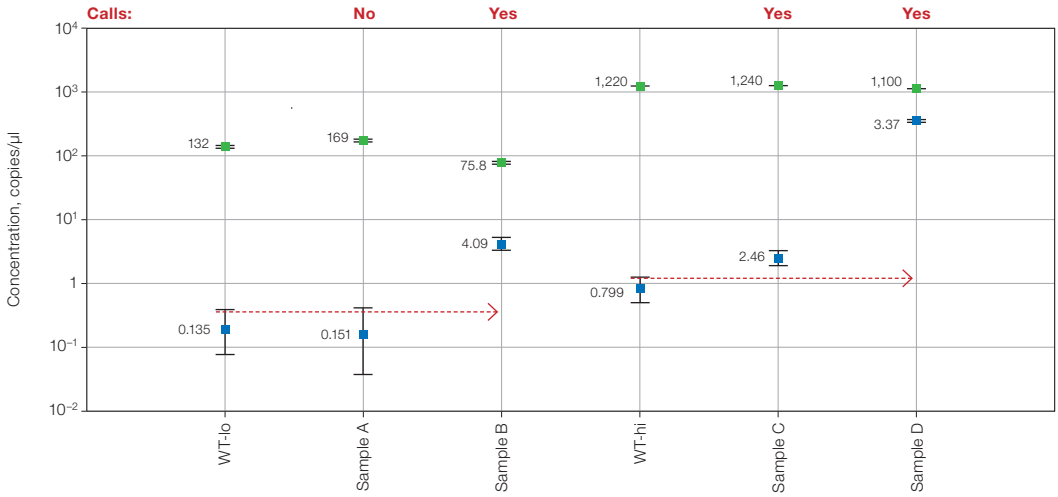

Fig. 16. Calling positive samples. The QuantaSoft Software concentration tab displays the concentration of the mutant allele and the 95% confidence intervals (error bars). If the upper error bar of the wild-type (WT)–only control (WT-lo and WT-hi) with a similar amount of DNA loaded per well as the sample does not overlap the lower error bar of the sample (red lines), then the sample is positive for the mutant. WT-hi and WT-lo are control template concentrations that flank the expected range of WT template concentration in the tested sample. WT template concentration (■); mutant template concentration (■).

Calling a Qualitative Positive Sample

There will sometimes be samples that lie right on the edge of a confident call. If possible, try running additional sample in order to screen more copies. If this is not possible, the borderline data can be assessed by comparing only the number of mutant single-positive droplets in sample wells with the number of FAM single-positive droplets in the WT-only (mutation-negative) control well, assuming loading is low (<20 ng/well) and comparable (Figure 17, A vs. B). For wells containing <20 ng sample per well, this can be done in QuantaSoft Software by reassigning double-positive droplets as HEX (WT) single-positive droplets. The assumption here is that at low loading, most double-positive droplets are the result of polymerase-induced errors. A statistical increase in mutant single positives here generally reflects the presence of a real mutant.

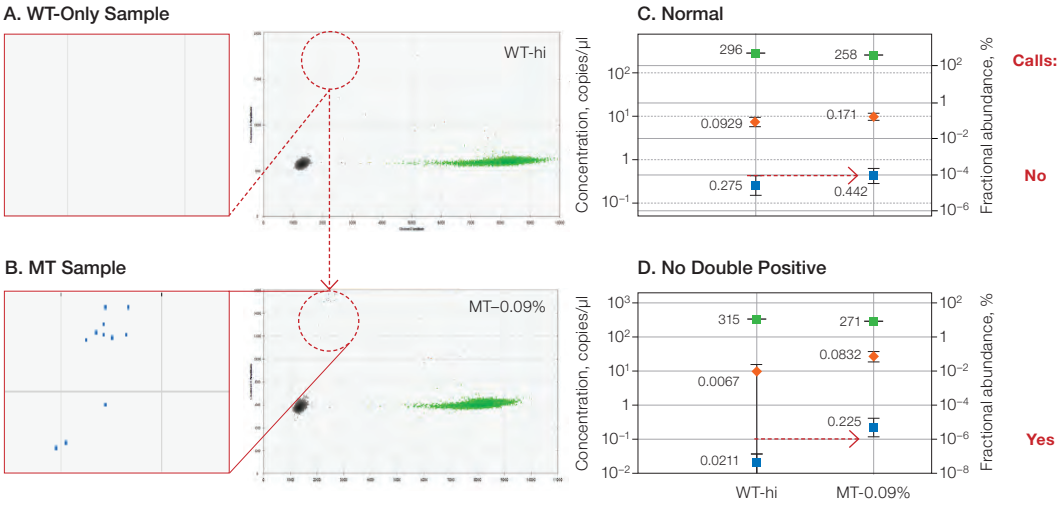

Fig. 17. Calling borderline samples in low DNA concentration wells. The wild-type–only (mutation-negative) control has few or no mutant single-positive droplets (**A**), while the mutant-containing sample (**B**) has many single-positive droplets. Rethresholding the double-positive droplets (orange) into single-positive wild-type droplets (green) allows a qualitative mutation-positive (blue) call to be made (**C** vs. **D**, red lines). WT-hi is a control template concentration that reflects the highest expected WT template concentration in the samples to be tested.

5 Troubleshooting and Frequently Asked Questions (FAQs)

1. When should I use multiple wells?

Multiple wells are needed when sample volume is relatively high but species of interest are present at low abundance. For example, a sample may contain a few copies of mutant *HRAS G12V* but was eluted in 50 µl final volume. Further concentration steps should be avoided in order to keep as many mutant copies as possible. The maximum sample volume in a 20 µl Droplet Digital™ PCR (ddPCR™) reaction is 8 µl, so at least seven reactions are needed to screen the full 50 µl of sample. For data analysis, all the reactions can be combined as if a single 140 µl reaction was used using the merge function of QuantaSoft™ Software.

One frequently asked question is if it is permissible to use one reaction and screen only 8 µl of that sample. By screening only part of the sample, you may suffer from sub-sampling error if the target is extremely rare. If you are operating in the range of 1–100 copies of your target, a small aliquot of that sample may not represent the true copy percentage since the target of interest is only present at a few copies in your sample. Thus, it is always recommended to screen a sample completely when the expected concentration falls into a range where subsampling error may significantly affect the accuracy of the experiment.

The merge-well function of QuantaSoft Software benefits rare event detection in many ways. It is a flexible way to adjust reaction numbers for samples with different volumes. An intrinsic property of end-point PCR is that it tolerates lower-efficiency assays, and thus single-copy detection is possible on the ddPCR platform but not on the real-time platform.

2. Can I use a prevalidated quantitative PCR (qPCR) TaqMan Assay for ddPCR?

Many TaqMan Assays that are designed for qPCR can be transferred directly to the ddPCR platform. It is recommended to run preliminary experiments with a temperature gradient in order to determine the optimum annealing temperature in the ddPCR supermixes. However, a subset of assays may not perform as expected and may require additional optimization.

3. What are causes of the rainy phenotype?

The following are factors that can cause rain:

Sample quality — fragmented or otherwise damaged DNA and/or the presence of PCR inhibitors

Sample type — nonhomogeneous sample with DNA modifications and single nucleotide polymorphisms

Template accessibility — local structures or other binding components mask the primer/probe binding region

Suboptimal assay design — T_m of the different oligonucleotides (primers and probes) are not compatible; nonspecific primers generating other off-target amplicons

Suboptimal thermal cycling — nonoptimized annealing temperature, annealing time, and/or cycle numbers

Operation and system — nonuniform droplet size and shredded droplets due to improper operation, handling, or system errors

4. I'm running duplex mutation detection assays and observing clusters close to the negative cluster but in places other than the WT or MT locations. What are these other clusters?

When running duplex mutation detection assays specific to one mutation when there are multiple possible variants of that mutation in the samples you are screening, you may see cross-reactive populations. In the example below, a *KRAS G12A* assay was used to screen samples. The sample came up negative for *KRAS G12A,* but the alternate cluster (red circle) indicates that this sample contains another, related *KRAS* mutation, likely with a one-base mismatch. It is acceptable to threshold these clusters as negative for your mutant of interest.

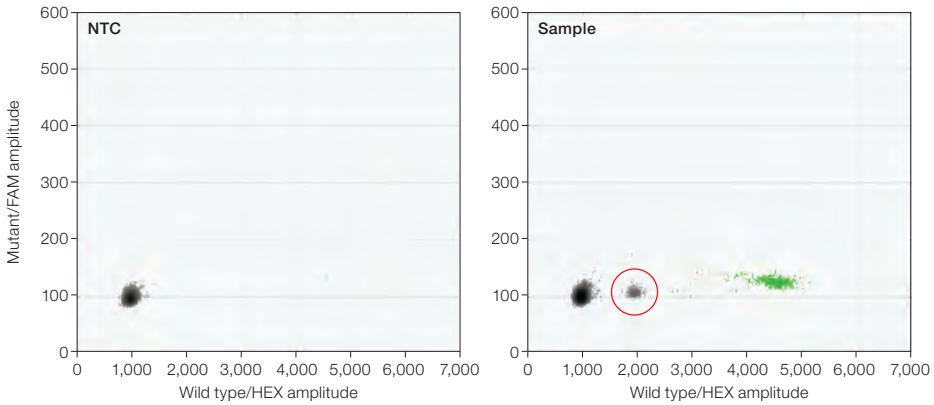

Fig 18. Cross-reactive populations are indicative of the presence of related mutation.

5. **Can I use the same WT control to screen multiple mutations at a given locus (for example, *KRAS G12D, KRAS G12V, KRAS G12A*)?**

   Each assay requires its own wild-type–only (mutation-negative) control. The WT-only control measures the false-positive rate (FPR) of that specific assay (primers and probe) on the WT DNA, and different mutations will lead to different FPRs. For example, C>T transitions in *KRAS G12D, G13D,* and *G12S* mutations have higher FPRs than *KRAS G12A* mutations.

6. **My assay runs at a different temperature and cycling protocol than the ddPCR recommendations. Can I run my assay the same as I do when using platforms other than ddPCR?**

   Certain parameters will change due to ddPCR chemistries and technology. Generally we recommend running a temperature gradient of your assays to determine the best annealing temperature for your assays in the Bio-Rad ddPCR Supermix. Primer concentrations are critical for ddPCR and running primers at much lower concentrations than we recommend will lead to loss of positive droplets (dropout), lower fluorescence amplitudes, and incorrect target quantification. Our cycling parameters are designed to be compatible with all of our assays for the most accurate quantification. Optimizing the cycling times and temperatures for assays other than ddPCR Assays is acceptable within certain limits. Droplets within a plate well will behave substantially differently than standard qPCR reactions and require additional time to reach the desired temperature. This is why we require altered ramp rates (~2°C/second) and longer annealing/extension times (minimum of 1 min).

A Appendix: Reaction Setup Using PrimePCR™ ddPCR™ Mutation Detection Assays

Reagent contents — the PrimePCR ddPCR Mutation Detection Assay is a 20x concentrated, ready-to-use primer-probe mix optimized for use with ddPCR Supermix for Probes (No dUTP). Each kit comes with 200 µl, 1,000 µl, or 2,500 µl of the 20x assay mix (900 nM primers and 250 nM for each probe, final concentration), sufficient for 200, 1,000, or 2,500 x 20 µl reactions, respectively.

Restriction digestion — DNA fragmentation by restriction digestion prior to droplet generation enables optimal accuracy by separating tandem gene copies, reducing sample viscosity, and improving template accessibility. Check the PrimePCR website (www.bio-rad.com/primepcr) for the optimal restriction enzyme to use with each assay. Restriction digestion of DNA samples can be done by direct spike-in of enzyme into the ddPCR reaction during setup (Table 5), or by conventional digestion prior to ddPCR according to manufacturer's instruction.

Uracil DNA glycosylase (UDG) treatment of formalin-fixed, paraffin-embedded (FFPE) samples — because formalin fixation of samples causes spurious C>T transitions, sample treatment with UDG is recommended. UDG removes templates containing uracils, which were created as a byproduct of cytosine deamination. UDG treatment is fast and inexpensive, and can be performed as a pretreatment or directly in the master mix.

Pretreatment: 10 µl FFPE sample plus 2 µl 10x UDG reaction buffer plus 2 units UDG. Incubate at 37°C for 20 min. In master mix: add 1 unit UDG to 20 µl reaction setup. Incubate at 37°C for 20 min prior to PCR.

Table 5. ddPCR reaction setup.

Component	Volume per Reaction, µl	Final Concentration
2x ddPCR Supermix for Probes (No dUTP)	10	1x
20x mutant target primers/probe (FAM)	1	450 nM primers/250 nM probe
20x wild-type primers/probe (HEX)	1	450 nM primers/250 nM probe
Restriction enzyme, in diluent* This component is necessary if the DNA sample is **not** predigested with appropriate restriction enzyme	1	Variable
DNA sample/water **,***	Variable	50 fg–132 ng
Final volume	**20**	**—**

* Approximately 2–5 units of restriction enzyme per 20 µl ddPCR reaction are recommended.

** DNA purification is not necessary if restriction digestion was performed before ddPCR reaction setup; however, the addition of restriction enzyme buffers with high salts can inhibit ddPCR and should be avoided. Use a minimum tenfold dilution of the digest to reduce the salt content of the sample.

*** Use 5–10 enzyme units per microgram of DNA, and 10–20 enzyme units per microgram of genomic DNA if separating tandem repeats.

ddPCR Reaction Setup

1. Thaw all frozen reaction components to room temperature. Mix thoroughly, centrifuge briefly to collect solutions at the bottom of the tubes, and then store on ice protected from light.

2. Prepare samples at room temperature. If multiple samples are to be assayed using the same target and wild-type assay, prepare a master reaction mix without sample template, dispense equal aliquots into the reaction tubes, and add the sample template to each reaction tube as the final step. Mix by pipetting up and down five to ten times to ensure even distribution of sample DNA. For most routine mutation detection applications, up to 130 ng of human genomic DNA can be added per ddPCR well for a final concentration of 2,000 copies per µl.

3. Vortex reaction mixture thoroughly, spin down, and dispense 20 µl of the mix into the sample well of the QX100™ or QX200™ Droplet Generator Cartridge. Follow general guidelines for droplet generation.

4. After droplet generation, transfer reaction mix into the recommended Eppendorf 96-well PCR plate. Program thermal cycling protocol on the C1000 Touch™ Thermal Cycler with 96–Deep Well Reaction Module according to Table 6.

5. Load the PCR plate into the thermal cycler and start the PCR run. After thermal cycling, transfer the PCR reaction plate into a QX100 or QX200 Droplet Reader and follow instrument-specific guidelines.

Table 6. Thermal cycling protocol.*

Cycling Step	Temperature, °C	Time	Ramp Rate	Number of Cycles
Enzyme activation	95	10 min	~2°C/sec	1
Denaturation	94	30 sec		40
Annealing/extension	55	1 min		
Enzyme deactivation	98	10 min		1
Hold (optional)	4	Infinite	~1°C/sec	1

* Use a heated lid set to 105°C.

B Appendix: Converting Nanograms to Copies for Human DNA

Table 7. Nanograms to copies conversion.

	Amount of Amplifiable DNA Analyzed per Assay, ng	QuantaSoft™ Software Readout, copies/µl	Haploid Genome Equivalents*	Maximum Sensitivity**
	400	6,060.6	121,212.1	0.002%
Maximum allowable DNA per well	350	5,303.0	106,060.6	0.003%
	300	4,545.5	90,909.1	0.003%
	250	3,787.9	75,757.6	0.004%
	200	3,030.3	60,606.1	0.005%
	150	2,272.7	45,454.5	0.007%
	100	1,515.2	30,303.0	0.010%
	75	1,136.4	22,727.3	0.013%
	50	757.6	15,151.5	0.020%
	40	606.1	12,121.2	0.025%
	33	500.0	10,000.0	0.030%
	25	378.8	7,575.8	0.040%
	20	303.0	6,060.6	0.050%
	15	227.3	4,545.5	0.066%
	10	151.5	3,030.3	0.099%
	5	75.8	1,515.2	0.198%
Very low DNA input	1	15.2	303.0	0.99%
	0.5	7.6	151.5	1.98%
	0.1	1.5	30.3	9.90%
Minimum copies	**0.01**	**0.2**	**3.0**	**99.00%**

* Assuming 3.3 pg DNA/haploid genome.
** Assuming no false positives and perfect controls.

The maximum sensitivity that can be attained by any assay is limited by the number of copies of a genome that are screened. This table converts the mass of DNA analyzed (nanograms) to copies of target analyzed, under certain assumptions. The first assumption is that one haploid human genome is 3.3 pg of DNA (C-value). The second assumption is that the QuantaSoft Software readout is in copies per µl (cpm) and that that concentration

multiplied by 20 µl per reaction gives the number of haploid genomes screened (third column). Finally, the third assumption is that three positive templates divided by the total haploid copies screened yields the potential maximum sensitivity possible (fourth column). This does not include real-world sources of error and is meant to serve as a guide.

C Appendix: Converting Molar Concentrations to Copies

Table 8. Molar concentration to copies.

Molarity to Copies/µl		QuantaSoft™ Software Readout, cpm	Moles to Copies	
Molarity	**Copies/µl**		**Moles**	**Copies**
1 M	6.02×10^{17}	3.01×10^{16}	1 micromole (10^{-6} moles)	6.02×10^{17}
100 mM	6.02×10^{16}	3.01×10^{15}	100 nanomole	6.02×10^{16}
10 mM	6.02×10^{15}	3.01×10^{14}	10 nmol	6.02×10^{15}
1 mM	6.02×10^{14}	3.01×10^{13}	1 nmol (10^{-9} moles)	6.02×10^{14}
100 µM	6.02×10^{13}	3.01×10^{12}	100 picomole	6.02×10^{13}
10 µM	6.02×10^{12}	3.01×10^{11}	10 pmol	6.02×10^{12}
1 µM	6.02×10^{11}	3.01×10^{10}	1 pmol (10^{-12} moles)	6.02×10^{11}
100 nM	6.02×10^{10}	3.01×10^{9}	100 femtomole	6.02×10^{10}
10 nM	6.02×10^{9}	3.01×10^{8}	10 fmol	6.02×10^{9}
1 nM	6.02×10^{8}	3.01×10^{7}	1 fmol (10^{-15} moles)	6.02×10^{8}
100 pM	6.02×10^{7}	3.01×10^{6}	100 attomole	6.02×10^{7}
10 pM	6.02×10^{6}	3.01×10^{5}	10 amol	6.02×10^{6}
1 pM	6.02×10^{5}	30,110.71	1 amol (10^{-18} moles)	6.02×10^{5}
100 fM	6.02×10^{4}	3,011.07	100 zeptomole	6.02×10^{4}
10 fM	6.02×10^{3}	301.11	10 zmol	6.02×10^{3}
1 fM	602	30.10	1 zmol (10^{-21} moles)	602
100 aM	60.2	3.01	100 yoctomole	60.2
10 aM	6.02	0.30	10 ymol	6.02

cpm = copies per microliter, assuming 1 µl in a 20 µl reaction.
General operating range of ddPCR in one well: 0.24–5,000 cpm.
1 M = 6.02×10^{23} copies/L = 6.02×10^{17} copies/µl.
1 mole = 6.02×10^{23} copies.
Avogadro's number: 6.02×10^{23}.

This table is a reference for converting moles or molar concentrations of stock into total copies and copies per µl (cpm) as shown in QuantaSoft Software. For the QuantaSoft column in the middle, it is assumed that 1 µl of stock is in a 20 µl Droplet Digital™ PCR (ddPCR™) reaction. The first two columns are molar concentrations and these are converted to total copies in the third column, in terms of copies/µl stock. On the right side are conversions of moles to total copies. The highlighted orange section indicates the general dynamic range of the system within one well.

D Appendix: Sample Plate Layout for Sensitive Limit of Detection

Table 9. Plate layout for sensitive LOD.

Master Mix	Starting	Final	1 reaction	NTCs 9 reactions	WT-lo + 0.004% MT 7 reactions	WT-hi + 0.01–0.1% MT 5 reactions	0.33–1% MT 2.5 reactions
Water			5 µl	45 µl	35 µl	25 µl	12.5 µl
2x ddPCR Supermix for Probes (No dUTP)	2x	1x	10 µl	90 µl	70 µl	50 µl	25 µl
20x mutant target primers/probe (FAM)	20x	1x	1 µl	9 µl	7 µl	5 µl	2.5 µl
20x wild-type primers/probe (HEX)	20x	1x	1 µl	9 µl	7 µl	5 µl	2.5 µl
Restriction enzyme		2–5 U	1 µl	9 µl	7 µl	5 µl	2.5 µl
Subtotal			18 µl	162 µl	126 µl	90 µl	45 µl
Wild-type template		2,000 cpm, 200 cpm	1.00 µl	—	7 µl	5 µl	2.5 µl
Mutant type template		1–0.004%	1.00 µl	—	7 µl	5 µl	2.5 µl
Water		Variable	—	9 µl	—	—	—
Final volume			20 µl	171 µl	140 µl	100 µl	50 µl

Plate Setup

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	WT-hi	WT-lo	0.01%	0.11%							
B	NTC	WT-hi	WT-lo	0.01%	0.11%							
C	NTC	WT-hi	0.004%	0.01%	0.11%							
D	NTC	WT-hi	0.004%	0.01%	0.11%							
E	NTC	WT-lo	0.004%	0.04%	0.33%							
F	NTC	WT-lo	0.004%	0.04%	0.33%							
G	NTC	WT-lo	0.004%	0.04%	1%							
H	NTC	WT-lo	0.004%	0.04%	1%							

Cycling Parameters

Temperature, °C	Time	Number of Cycles
95	10 min	1x
94	30 sec	
55	1 min	40x
98	10 min	
4	Hold	1x

20x primer-probe mix: 18 µM PCR primers (each), 5 µM probe.

Final concentrations: 900 nM PCR primers (each), 250 nM probe.

Recommend 20 µl per PCR reaction. Prepare master mix for an additional 0.5–1 well so that 20 µl droplets can be used in droplet generator cartridge.

E Appendix: False-Positive Rate Changes with DNA Amount

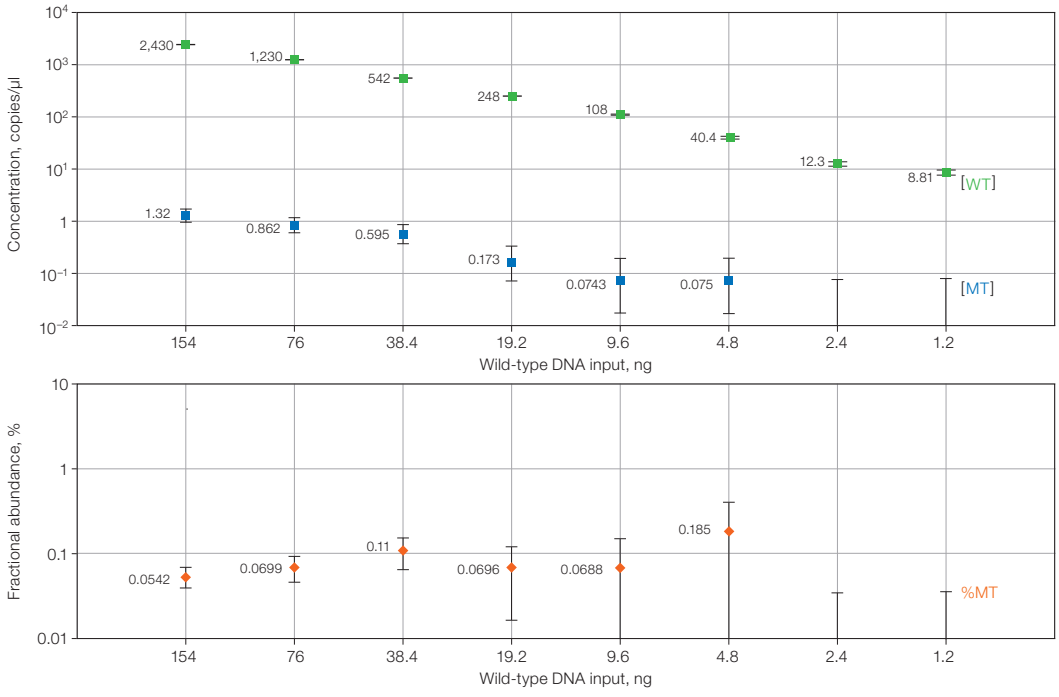

Fig. 19. False-positive rate (FPR) changes with DNA amount. A twofold dilution series of wild-type (WT) DNA was assayed for the mutant (MT) concentration (FPR) detected. The concentration of MT copies in wild-type–only DNA changes with the amount of WT DNA loaded. This is not a simple, linear relationship and statistical error can play a significant role. This is why we recommend that WT controls are added at an amount that mimics the samples tested. WT template concentration (■); MT template concentration (■); MT fractional abundance (◆).

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