

The Digital MIQE Guidelines Update: Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020

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Special Report

The Digital MIQE Guidelines Update: Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020

The dMIQE Group*

ABSTRACT: Digital PCR (dPCR) has developed considerably since the publication of the Minimum Information for Publication of Digital PCR Experiments (dMIQE) guidelines in 2013, with advances in instrumentation, software, applications, and our understanding of its technological potential. Yet these developments also have associated challenges; data analysis steps, including threshold setting, can be difficult and preanalytical steps required to purify, concentrate, and modify nucleic acids can lead to measurement error. To assist independent corroboration of conclusions, comprehensive disclosure of all relevant experimental details is required. To support the community and reflect the growing use of dPCR, we present an update to dMIQE, dMIQE2020, including a simplified dMIQE table format to assist researchers in providing key experimental information and understanding of the associated experimental process. Adoption of dMIQE2020 by the scientific community will assist in standardizing experimental protocols, maximize efficient utilization of resources, and further enhance the impact of this powerful technology.

Introduction

Since the publication of the guidelines for the Minimum Information for Publication of Quantitative Digital PCR Experiments (dMIQE) *(1)*, digital PCR (dPCR) has seen considerable technological development with a plethora of new applications. dPCR has progressed from an expensive approach with a limited application niche, available to only a few laboratories, towards a mainstream global technology *(2)* offering unique advantages and applications to many scientists.

To reflect this advancement, we present an update to dMIQE, dMIQE2020, which builds on the original guidelines to account for the increase in the number of applications and new platforms that have become available in the last 7 years. We highlight some of the associated advantages and limitations, and these updated guidelines are written with the support of dPCR instrument manufacturers (see Acknowledgment section). The intention is to enable dMIQE2020 to reflect this maturing technology by addressing new factors that need to be included in publications reporting dPCR data. We also present a revised simplified dMIQE table format (Supplemental Table 1) to aid application and increase adoption and provide an example of a completed table (Supplemental Table 2).

Description of the Method and Brief History

A dPCR reaction is performed using limiting dilution to separate the nucleic acid molecules amongst a large number of subreactions, termed partitions (Table 1 provides a summary of the terminology for dPCR). This 'partitioning' capitalizes on the random distribution of nucleic acid molecules in solution, so that some of the partitions contain single (or few) copies of target molecules and, importantly, some contain none. Following partitioning, the reaction, comprising all the partitions to be analyzed, is subjected to PCR. Each dPCR partition contains the core reagents used in a real-time quantitative PCR (qPCR) reaction that will generate a fluorescent signal in response to the presence of a target sequence. A positive partition is identified by an increased fluorescent intensity compared to a negative partition with baseline signal only. Quantification is performed by applying Poisson statistics to the proportion of the negative partitions (typically calculated by subtracting the number of positive partitions [k] from the total number [n], see Equation 2 below) to account for positive partitions that initially contained more than one target molecule.

The concept of dPCR was developed before qPCR. As early as 1988, just a few years after PCR was described, Saiki et al. *(3)* applied limiting dilution to the PCR, which was subsequently used to detect HIV DNA *(4)*. In the early 1990s, several articles were published applying the concept mainly in the areas of virology, lymphoid biology, and neoplasia *(5)*. However, following the initial description *(6)* and subsequent development of qPCR, offering increasingly affordable, precise, high-throughput, and multiplexed measurement of

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nucleic acids, resource intensive 'limiting dilution PCR' was eclipsed.

Development of the method did not completely stop, however, with the term 'digital PCR' being coined by Vogelstein and Kinzler in 1999 *(7)* as a method that offered improved analytical sensitivity when measuring mixtures of single nucleotide variants by PCR. This concept was further advanced by developing BEAMing *(8)* and its potential highlighted with some of the earliest circulating tumor DNA (ctDNA) measurement in cancer patients *(9)*. More recently, advances in microfluidics have enabled the development of instruments that can partition in very small volume formats. Partitioning is currently achieved either through the generation of water-in-oil droplets or using prefabricated chips that contain solid chambers into which the reaction is loaded.

Another development has been the establishment of instruments with greater than 10,000 partitions per reaction. These instruments can analyze tens of samples in a single run typically providing 2- or 3-color formats allowing for multiplexing, with more colors promised by manufacturers for future instruments. This contributes to improved precision (Fig. 1A), increased dynamic range (Fig. 1B), and increased analytical sensitivity due to the larger volume of nucleic acids extract analyzed per reaction.

In parallel with hardware development, the expansion of software tools (both vendor-specific and

Table 1. The meaning of frequently used terminology when discussing dPCR.

dPCR term	Description	Alternative name
prereaction	the initial volume prepared prior to partitioning that contains master mix, dNTPs, assay, and template	
partition	the subreaction used for limiting dilution and subsequently measured as positive or negative post reaction	droplet, chamber[a]
n	the total number of partitions used for quantification	accepted partitions /droplets/ chambers[a], analyzable partitions/ droplets/chambers[a]
V_p	the volume of each partition	
reaction	the total volume of the measured partitions (n x V_p)	
dead volume	difference between the volume of the prereaction and reaction	
k	the number of positive partitions in a reaction used for quantification	
w	the number of negative partitions in a reaction used for quantification	
Poisson	the statistical distribution used to account for the probability of a partition initially containing more than one target	
λ	lambda, the average number of target molecules per partition	
fluorescence intensity	the fluorescence of a partition	fluorescence amplitude, endpoint fluorescence, relative fluorescence unit
baseline	the fluorescence of the negative partitions	fluorescence noise/ background
peak resolution	a measure of the separation in fluorescence between positive and negative partitions	separability score, amplitude
threshold	the line that separates the partition clusters based on amplitude	
cluster	the group of partitions that are located in a similar space within a scatter plot based on amplitude	population, droplet population
rain	the partitions that are located within the space between the positive and negative clusters	
higher order multiplexing	the term given to multiplexing that can count more targets than there are fluorescent detection channels	intensity multiplexing

[a]The term 'chambers' is used to define the partitions of instruments that use prefabricated chips, however this term is also used by some manufacturers to describe the reaction vessel. Consequently, the use of the term 'partition' to describe dPCR subreactions is recommended as it is not used elsewhere when describing the technology and is agnostic to the partitioning format of a given instrument.

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platform-independent) has given operators greater control of their analysis. Scatter plots that depict the endpoint fluorescence of each partition are now commonly available. These plots also enable quality control checks to be performed in parallel for individual reactions and whole experiments. Other developments include the ability to define thresholds that optimally separate positive and negative partitions, to combine different reactions for greater sensitivity and higher precision, and to export data to enable further analysis using third party software.

Despite the increasing capabilities and use of dPCR, many biological and clinical research scientists are unclear as to what the practical advantages are. Placed in the same methodological space as established qPCR and the increasingly powerful massively parallel (or next generation) sequencing, this is perhaps unsurprising. The reality is that dPCR provides a number of unique opportunities. Here we explore recent developments and applications of dPCR, discuss the factors that should be considered during the experimental design, and detail considerations to be included in publications reporting dPCR results.

An Overview of the Applications of dPCR

Recent uses of dPCR have spanned numerous DNA, RNA, and epigenetic applications. A popular use of the method is the detection and quantification of rare genetic variants (e.g., single nucleotide variants) in mixtures of other, more predominant, variants of the same sequence. Such 'rare' sequence detection can measure actionable mutations in ctDNA *(10)*, fetal genetic variants in noninvasive prenatal testing *(11)*, polymorphisms of a donor organ as an assessment of potential graft rejection *(12, 13)*, as well as rare bacterial genotypes *(14)* and viral drug resistance *(15)*. An example of early direct clinical diagnostic application is the measurement of ctDNA in liquid biopsies to guide the treatment of non-small cell lung cancer *(16, 17)*.

dPCR can offer greater precision than qPCR *(18)* and is far simpler to use for copy number quantification due the binary nature in which the partitions are counted as positive or negative. The increased precision of dPCR *(18)* enabled improved measurement of copy number variants *(19, 20)*, including in gene amplification in neuroblastoma *(21)* and fetal trisomy by noninvasive prenatal testing *(22)*. dPCR also allows rare event or trace level detection with high confidence since only a single or small number of DNA molecules are amplified in each individual partition, regardless of whether an experiment has 10 or 10 000 target molecules per reaction. While qPCR can detect very low concentrations of a target, calibration of trace measurements is challenging. This is one of the reasons dPCR has been explored as a method for trace level measurements in minimal residual disease *(23, 24)* and latency in viral infections such as HIV *(25–27)*.

The analytical sensitivity of the measurement of double stranded DNA molecules can be further enhanced by denaturing the molecules prior to partitioning *(28)*. Since single strands end up in different partitions, the analytical sensitivity is improved by a

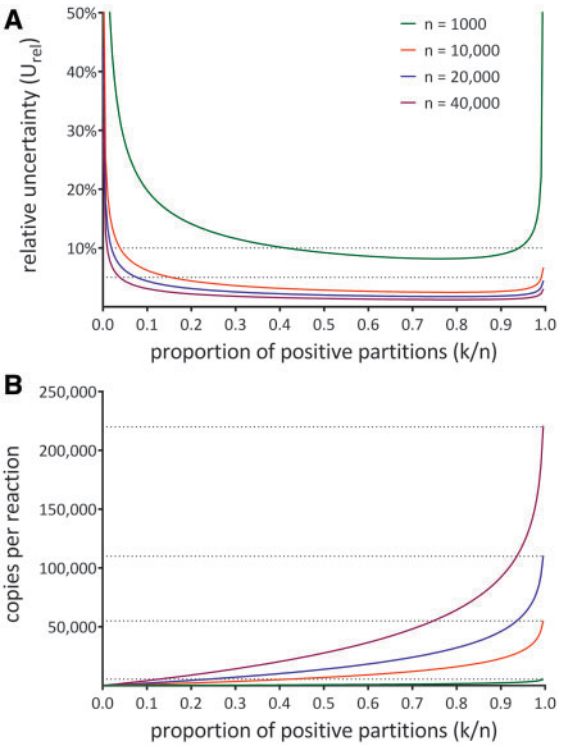


Fig. 1. Predicted precision and linear range of the Poisson distribution. Each graph is generated mathematically based on the Poisson distribution for dPCR reactions with 4 different numbers of partitions (n). The majority of dPCR instruments offer reactions where n > 10 000. (A) The relative uncertainty (based on modeling the 95% upper confidence limit of λ) is highest at the extremes of the range (very few positive partitions; k/n < 0.1 or very few negative partitions; k/n > 0.95). As the number of partitions in a reaction increases, so does the precision at a given proportion of positive partitions. Once n > 10 000 the relative uncertainty is <5% for the majority of the range. Horizontal dotted lines correspond to a relative uncertainty of 5% and 10%. (B) The dynamic range is proportional to the number of partitions in a reaction, note the dynamic range is greater than the number of partitions. The horizontal dotted lines correspond to the copies per reaction for each given n where the relative uncertainty of λ is less than 50%.

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factor of two. Other applications that exploit the unique partitioning of dPCR include cis-trans linkage relationships between two targets *(29–31)* and 'drop-off' assays for identifying the frequency of a mutation of unknown sequence *(32),* and evaluation of gene editing efficiency when using approaches like CRISPR-Cas9 *(33)*.

Furthermore, dPCR provides high reproducibility to the above technical advantages. This is possible when the same target is measured in different laboratories *(34, 35)* using different assays or assay formats *(36)*, or instruments from different manufacturers *(37, 38)*. This is also achievable both when measuring purified nucleic acid, but also whole biological samples in which preanalytical steps such as extraction need to be included *(36, 39)*. This characteristic has made dPCR a popular method to quantify reference materials *(40, 41)*, to support applied molecular testing in clinical diagnostics *(42–44)* and food testing *(45–47)*.

When used to conduct quantitative measurements, molecular genetic methods have historically applied mass and mole, combined with volume, to calculate copy number concentration. Mass or mole are arguably not ideal when considering a large macromolecule such as DNA, and nucleic acid calibration materials have rarely been traceable to the International System of Units (SI) *(48)*. dPCR has the capability of counting all intact (equal or larger than the amplicon) DNA molecules containing a specific target sequence *(49)*, thereby potentially offering SI traceability via counting to the unit one *(48)*. To maximize the potential impact of such a capability, efforts have been made to harmonize and standardize best practices in dPCR (and qPCR) in the ISO 20395 standard *(50)*.

Quantification accuracy for copy number measurements is dependent on both completeness of molecular count and accurate definition of the unit volume of sample and total reaction (i.e., number of partitions of accurately defined volume). Both need to be demonstrated for claims of SI traceability by dPCR to be supported. International collaboration among national metrology institutes, supported by the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM), have led research demonstrating that dPCR can indeed measure with sufficient accuracy for primary SI-traceability *(38, 51)*. dPCR has provided the first ever nucleic acid reference measurement procedure accepted by the Joint Committee on Traceability in Laboratory Medicine (JCTLM) *(38)* and dPCR is included as an example of a higher order reference measurement procedure within the new edition of the ISO 17511 guideline on metrological traceability of values assigned to calibrators and control materials for diagnostic methods *(52)*.

The potential for dPCR to enable new research opportunities and to support traceability in the wider field of molecular genetic measurement should have a major impact on the accuracy of nucleic acids measurement as a whole. However, researchers and manufacturers must tread cautiously to ensure that the nuances that may affect these measurements are understood. What follows are some steps to consider on this journey.

Considerations for Designing and Performing dPCR Experiments

Like qPCR, to maximize the performance of dPCR, specific considerations are required to ensure unbiased and reproducible measurements *(50, 53)*. The following sections outline how best to design and perform dPCR experiments. Sections may be interdependent: for example, to determine the false positive rate of an assay, not only assay design, but selection of control materials and partition classification are important.

Following dPCR, the end-point fluorescence of target containing positive partitions is higher fluorescence than those without, negative partitions (background). These fluorescence values can be plotted in one-color dimension with the fluorescence intensity against partition number (Fig. 2) or as two-colors (Fig. 3A–D) or more (Fig. 3E), with the fluorescence intensity of the different channels aligned to different axes. Visualization of these plots can aid assay optimization and quality control.

ASSAY DESIGN AND OPTIMIZATION

dPCR requires the same careful assay design considerations as those required for qPCR *(53, 54)*. Smaller (<150 bp) amplicons are desirable although larger products can also be quantified *(55)*. To generate the fluorescent signal by PCR, probe-based chemistries (e.g., hydrolysis probes, ideally with nonfluorescent quenchers) or DNA binding dyes (e.g., EvaGreen) can be used when compatible with the dPCR platform.

Assay optimization, by varying the annealing temperature (Fig. 2A) as well as primer and probe concentrations (Fig. 2B), is best performed using a template that closely matches that of the test template, as performance may vary with different template types (e.g., plasmids, genomic DNA, cell-free DNA, synthetic DNA). Furthermore, while different batch syntheses of probes of the same sequence can change the final fluorescence intensities, this can have a negligible effect on the measured results (Fig. 2C). Matrix effects and inhibitors may also reduce the fluorescence intensity of the positive partitions. While 'suboptimal' reactions may provide similar results to more optimum conditions (Fig. 2D), reflecting methodological robustness, optimization

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should strive to elevate the positive from the negative populations, to maximize the 'peak resolution' *(56)* and reduce the number of partitions in between that are termed 'rain' (see below).

MULTIPLEX ASSAYS

Design criteria for multiplex dPCR are similar to other multiplex PCR applications. The increased number of primers and probes requires additional consideration of complementary sequences to avoid nonspecific hybridization. Similar to qPCR, multiplexing is performed with fluorescently-labeled probes that are preferentially detected in different fluorescence channels (Fig. 3).

The simultaneous analysis afforded by multiplexing can improve resource efficiency, reduce the amount of sample needed for analysis (important when the specimen is limiting), and allow for direct internal control of an individual reaction. Multiplex dPCR also has other advantages; for copy number variants, the pairing of target and reference genes to determine their ratio arises naturally using a duplex approach (Fig. 3A) *(57, 58)*. Similarly, for biallelic variation of single nucleotide variants or small insertion/deletions, typically analyzed with two hydrolysis probes, a duplex approach visualized using two color plots is the favored format (Fig. 3B). In drop-off assays *(59)*, genotype is determined by counting partition numbers from the single and double positive clusters (Fig. 3C); it is not possible to make this calculation accurately using single color analyses.

Two color analysis can also be applied to identify some technical artifacts that may be difficult to discern with single color analysis. For example, fluorescent bleed through (also termed crosstalk or spillover) occurs when signal from one fluorophore is detected in a channel intended for another. If a duplex experiment is visualized as respective single color plots, fluorescent bleed through may appear as additional clusters and can be mistaken for reduced analytical specificity (Fig. 3A; see red arrow). In multiple color plots, fluorescent bleed through clearly manifests as a 'leaning' or 'lifting' of the single positive partitions away from the intended axes towards the expected position of the double positive partitions (Fig. 3A).

Fluorescent bleed through can be determined by performing single probe reactions, using a template that only contains the intended target, and visualizing the experiment in two colors. If the clusters 'lean' or 'lift' into the other axis, then bleed through is confirmed. Reducing the concentration of the relevant probe, changing the fluorophore, and/or applying a color compensation matrix may reduce the bleed through.

Bleed through can often be tolerated as long as its source is understood. If the 'lean' or 'lift' is absent when using a single color reaction, then it suggests that this may be caused by something else, such as reduced assay specificity. Probes designed to similar sequences (e.g., single nucleotide variants) may bind to the alternate variant, reducing specificity *(60)*. While this may be minor, a 'lean' or 'lift' similar to fluorescence bleed through may occur (Fig. 3B), reducing the peak resolution for the affected reporter. When an alternative variant is predominant, as in rare variants within cell-free DNA, false positive signals caused by factors like PCR errors, will ultimately limit the lower fractional abundance that can be measured (see further and Fig. 4).

Another artifact with competing probe duplexes *(59)* is a drop in fluorescence in the double positive partitions (Fig. 3B). When variants of the same molecule are amplified by a single primer pair in the same partition, competitive PCR or partition-specific competition (PSC) occurs *(59)*. Where different variants are both present in a partition, they will compete for the primers, resulting in a reduced fluorescence intensity when compared to partitions containing a single variant. PSC can occur even when the reaction is performed in singleplex because if other variants (or pseudogenes with sufficient homology) are present, they will amplify and compete with the variant of interest reducing peak resolution. If the method is performed in duplex and evaluated in a two-color plot, it is clear that PSC is occurring and thresholds are easier to set (Fig. 3B). In drop-off assays, PSC can cause the double positive partitions to appear as a second cluster with reduced fluorescence intensity (Fig. 3C). The impact of PSC, fluorescent bleed through, and other factors can be evaluated using an optimization and quality control protocol outlined previously *(59)*.

In addition to multicolor multiplexing, dPCR can specifically detect more than one target within an individual fluorescent channel. This 'higher order multiplexing' is achieved by varying the concentrations of different probes using the same fluorophore *(61)* (Fig. 3D) or differently sized amplicons with DNA binding dyes *(62)*. Higher order multiplexing can be performed in a single color, where two different targets are detected within a single reaction *(63)*, or in multicolor assays that use two or more probe colors to detect more targets than fluorophores with a single reaction *(61)*.

TEMPLATE

As with all other PCR formats, DNA is the only nucleic acid that can be measured by dPCR. Like qPCR, template complexity may impact assay performance as seen with circular plasmids and high fragment length genomic DNA *(37)*. Restriction digestion to small linear DNA fragments may equalize template differences *(36, 49, 64)*, and prevent underestimation of linked target molecules *(18)*. However, restriction sites must not be

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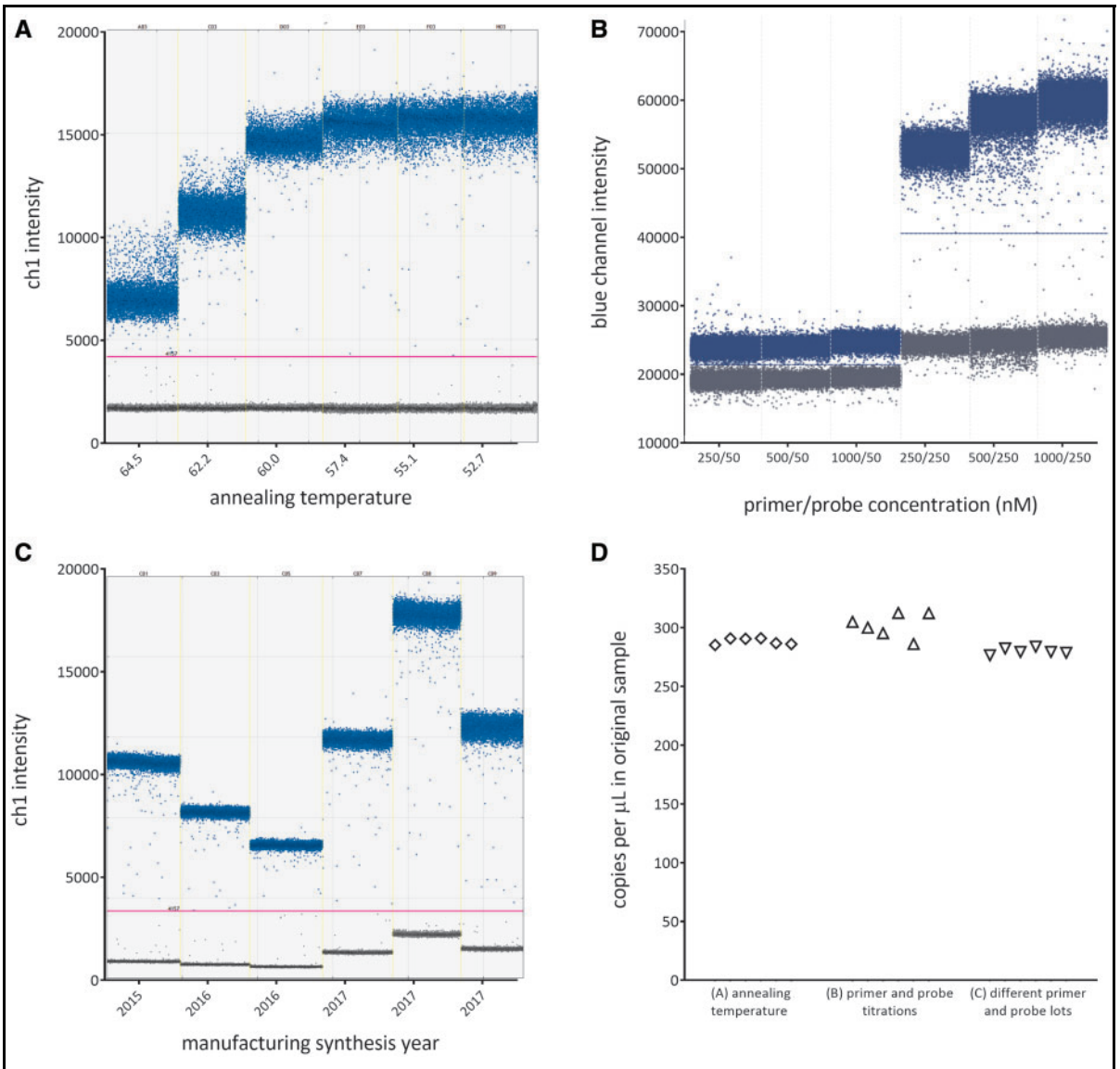


Fig. 2. Examples of the one color plot outputs demonstrating methodological robustness. The same assay and genomic DNA template is used in all parts of the figure. Unless otherwise stated, each reaction contains 900nM of each primer and 250nM of probe, annealing temperature: 60C, run for 40 PCR cycles. (A) The effect of the annealing temperature (gradient 65C to 52C from left to right) on the final fluorescence intensity of the positive and negative partitions. Each reaction contains approximately 20000 copies of the gDNA template analyzed using the QX200 (Bio-Rad) with Supermix for probes (no dUTP) (Bio-Rad). The horizontal pink line represents the threshold to separate the positive and negative partitions. (B) The effect of primer and probe concentrations on the difference in fluorescence intensity between positive and negative partitions. Reactions 1-3 have 250, 500, and 1000nM of primer, respectively, all with 50nM of probe. Reactions 4-6 have 250, 500, and 1000nM of primer, respectively all with 250nM of probe. Each reaction contains approximately 40000 copies of the gDNA template analyzed using the Naica (Stilla) and Perfecta Multiplex qPCR Toughmix (Quanta BioSciences). The blue horizontal lines represent the threshold to separate the positive and negative partitions. (C) The difference in fluorescence intensity of positive and negative partitions between 6 different batches of the same assay purchased between 2012 and 2017 from 3 different manufacturers. The genomic DNA template concentration and dPCR platform is the same as that described in (A). (D) Comparison of the copy number concentrations calculated from each of the 18 reactions shown in (A)-(C). Each reaction is represented by a symbol in the order it appears in its respective color plot.

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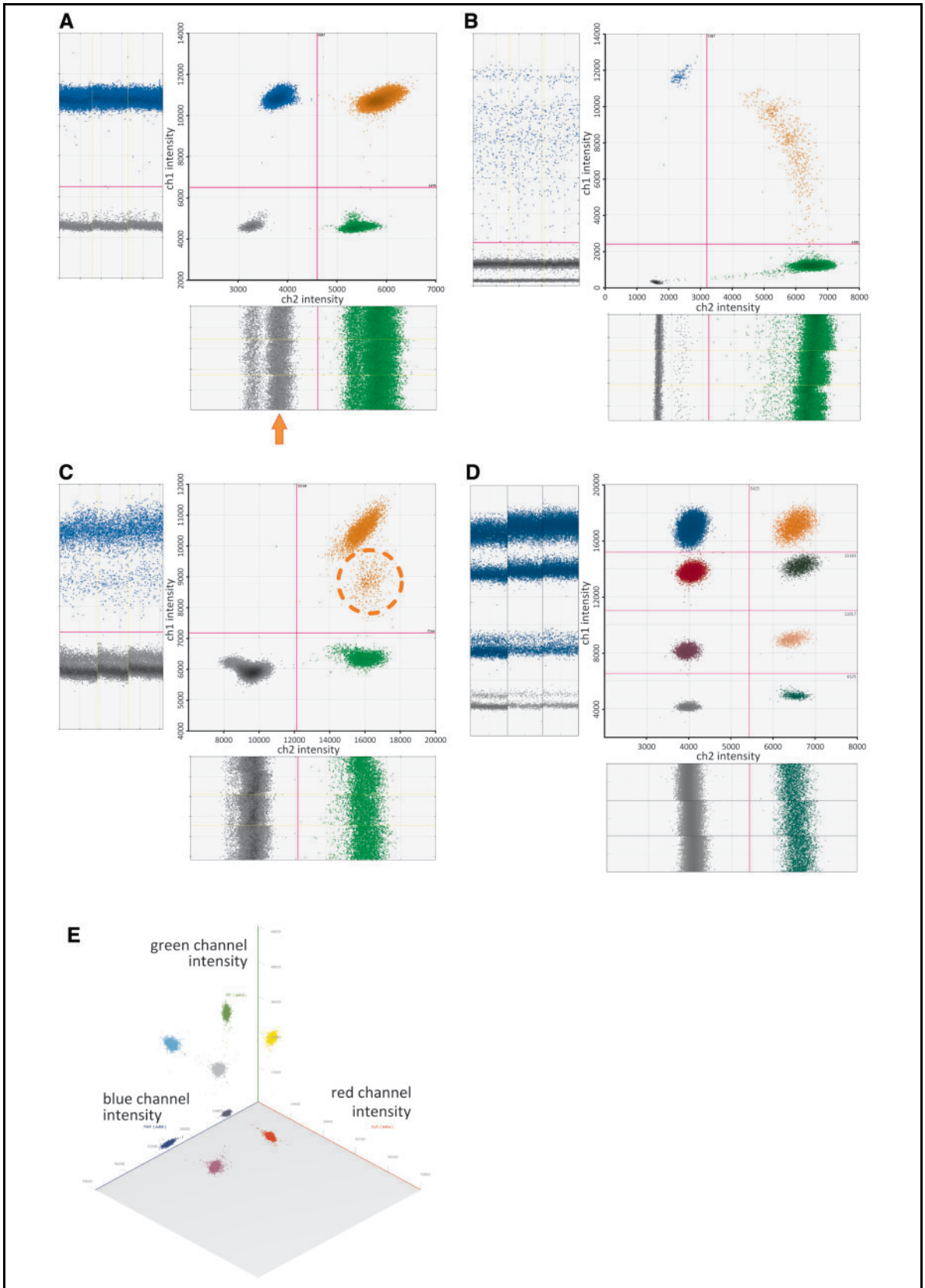


Fig. 3. Examples of two and three color plot outputs using different multiplex format demonstrating leaning, lifting and partition specific competition.

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Fig. 3. (Continued) Parts A-D of the figure have been generated using the QX200 (BioRad); each part shows a two color scatter plot with the corresponding single color plots orientated along the corresponding axis (channel 1 (Ch1); y-axis, and channel 2 (Ch2); x-axis). The horizontal and vertical pink lines represent the thresholds for Ch1 and Ch2, respectively. Part E of the figure has been generated using the Naica (Stilla); a three color plot is shown with the three axes orientated as the blue channel (x-axis), red channel (y-axis), and green channel (z-axis). The 3 thresholds are not displayed for aesthetic purposes. The corresponding two color plots are presented in Supplemental Fig. S2. (A) Noncompeting duplex reaction (for definition of multiplex formats see *(59)*) containing separate primer pairs for each amplicon. The ratio between the 2 targets is ~1.6. The partition clusters are colored based on their classification: blue; Ch1 positive only, green; Ch2 positive only, orange; double positive (both Ch1 and Ch2) and gray; double negative. Example of fluorescent bleed through is evident with the 'leaning' of the blue cluster in the two color plot that appears to show additional negative cluster (red arrow) in the green single color pot. (B) Competing duplex reactions containing a single primer pair with different probes targeting two different single nucleotide variants. The mutant target (blue) is approximately 1% of the wild type target (green). The clusters are colored as described in (A). Evidence of PSC is shown in the orange cluster that forms an 'arc' instead of the defined orange cluster observed in (A). Indication of reduced probe specificity is shown as the 'leaning' and 'lifting' of the blue and green clusters, respectively. (C) Drop-off assay containing a single primer pair with different probes that bind to different region of the same amplicon. The two targets are in approximate equal numbers. The clusters are colored based on their classification; variant only (green), wild type and double positive (orange). Evidence of PSC is shown with a smaller orange cluster (within dotted red circle), this cluster contains *wt* and variant double positives partitions while the larger orange cluster contains *wt* partitions only. (D) Higher order multiplexing reaction to measure three targets in 2 fluorescent channels; 2 targets (T1 and T2) in Ch1 and 1 target (T3) in Ch2. Each is with a different primer pair and at approximately 1:2:1 ratios as T1: T2: T3. Clusters are colored based on their classification: purple; T1 positive only, red; T2 positive only, green; T3 positive only, pink; T1 and T3, black; T2 and T3, blue; T1 and T2, orange; T1, T2 and T3. (E) Three color non-competing triplex reaction containing separate primer pairs for each amplicon. The ratio between the 3 targets is ~1. The partition clusters are colored based on their classification: dark blue; blue positive only, green; green positive only, red; red positive only; cyan; double positive (both blue and green), yellow; double positive (both green and red); purple; double positive (both blue and red), light grey; triple positive (blue, green and red) and dark grey; triple negative. Fluorescent bleed through has been corrected using an assay specific compensation matrix experiment as recommended by the instrument manufacturer.

present in the amplicon sequence, and digestion of already fragmented DNA (e.g., from formalin-fixed, paraffin embedded tissues or cell-free DNA) may result in a loss of signal *(36)*.

RNA transcripts can only be measured by first converting to complementary DNA (cDNA) in reverse transcriptase digital PCR (RT-dPCR) using either one- or two-step formats. In a one-step strategy, RNA is partitioned with both reverse transcription and PCR occurring sequentially in the same partition. Even if multiple cDNA copies are generated from each RNA molecule, results are not overestimated. In two-step reactions, reverse transcription is first performed in bulk before partitioning the cDNA and subsequent dPCR in a separate reaction. The reverse transcriptase step can be a predominant source of error that should be considered during experimental design *(65)*.

While newer dPCR formats have greater dynamic ranges, they cannot compete with qPCR, which is typically capable of over 6 orders of magnitude. Consequently, in certain situations a prior knowledge of the template concentration may be required to avoid saturating the instrument. When concentrations are sufficiently high, commonly used methods, such as those that employ fluorometry and spectrophotometry, can be used to quantify nucleic acids and guide the dilution to concentrations for optimal measurement using dPCR. It should be noted that such methods estimate mass per unit volume of the component nucleic acid bases not the macromolecule. Consequently, determination of genome copies using approaches that measure mass requires knowledge, or assumptions, of template composition, purity, and quality to convert mass to moles. Users should also be aware of potential interfering factors that may disrupt the accuracy of such optical methods. When comparing mass-based nucleic acid quantification with dPCR results, or those from any method used to calculate molecular copy number, a clear description of the molecular weight of the genome used to calculate the genome equivalents must be included, along with the method used to calculate this value.

PREANALYTICS

While dPCR can be accurate, performance depends on the amount of template added to the reaction. Error

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introduced during steps prior to dPCR (such as from extraction, template modification, or reverse transcription) will impact on results. To describe error, we refer to the International Vocabulary of Metrology *(66)* where error may be random (impacting on precision) or systematic (impacting on trueness leading to bias). dPCR can be highly precise with the potential to measure minute differences. However, if the preanalytical error is large, accuracy (closeness of agreement between measured quantity value and the true quantity value *(66)*, in terms of both precision and bias) will be compromised and incorrect conclusions generated. This may manifest in a dPCR reaction as a result of both suboptimal quantity and quality of the extracted nucleic acids.

Therefore, the preanalytical precision and bias of any dPCR assay should be measured and, where appropriate, incorporated into the conclusions. Replication of the whole procedure (e.g. by repeating the extraction on multiple samples) should be used to estimate random error. Process controls for specific protocol steps may be necessary to reveal systematic errors related to extraction, enrichment, modification, and reverse transcription. Conveniently, the precision of dPCR makes it an ideal tool to assess preanalytical procedures, and has been applied to nucleic acid isolation *(36)*, massively parallel sequencing library preparation *(67, 68)*, reverse transcription efficiency *(69)*, preamplification *(57)*, and genome editing using methods like CRISPR-Cas9 *(70)*.

EXPERIMENTAL CONTROLS AND CALIBRATION

Although dPCR is far simpler to calibrate than qPCR, it should not be assumed to be 'calibration free'. External calibration may be necessary to identify biases in preanalytical steps and where used, calibrators should perform similarly to the samples of interest. Internal calibration, or normalization, is often used by reporting results as percentages, ratios, fractional abundances, or normalized copies. For ctDNA, variants are often expressed as a variant allelic frequency or fractional abundance relative to the sum of both wild type and variants sequences. However, even in this case, reporting the absolute DNA concentration is also important because it may directly affect the observable false positive rate (Fig. 4) and provides an idea of what can be expected in terms of precision and analytical sensitivity. Although normalization to total nucleic acid concentration may be appropriate, other denominators can be used, such as biofluid volume used historically for viral load measurements and increasingly in oncology for ctDNA measurement. Importantly, the identity and rationale for control and/or calibration strategy should be clearly described.

A variety of control reactions are necessary in dPCR. Negative control reactions that contain the reaction mix, including primers and probes, but do not the nucleic acid template can identify cross contamination between samples, as well as carry-over contamination from previous amplified product. If DNA concentrations are high, a few negative controls may be adequate to monitor for cross contamination. However, for low copy number 'trace' analysis, a larger number of negative controls randomly included amongst the samples of interest will have a better chance of identifying results that are derived from false positive partitions.

An equally important negative control for inclusion in dPCR experiments is the same DNA/RNA background, but without the targeted sequence. These controls can assess specificity as well as contamination. This is particularly important when measuring rare genetic variants in samples that contain predominantly wild-type sequence. The frequency of low level false positive results (Fig. 4) defines the limit of detection or quantification and may aid in the setting of thresholds. Furthermore, when measuring real samples, extract quality can impact on the fluorescence intensity which can lead to variations in peak resolution.

While negative controls are essential, positive controls that ideally reflect real samples in complexity, integrity, purity, and concentration are also important. dPCR is less susceptible to inhibition, but is not immune *(71)*. Internal positive controls *(50)* confirm negative results and are essential when dPCR is used in clinical diagnostics. Positive controls (both internal and external) of defined concentration provide quality assurance and are particularly useful when evaluating the preanalytical steps. The number of positive partitions in low-level trace analysis may be so low that the appropriate threshold setting is best determined from a more concentrated positive control.

Considerations for Data Analysis

For dPCR results to follow the Poisson distribution, a number of conditions must be met *(60, 72)*. There needs to be 1) clear discrimination between positive and negative partitions; 2) partitions of equal defined volume; and 3) random distribution of target molecules among partitions. These conditions are not always met in practice, and consequently each step of the dPCR data analysis pipeline should be described *(60, 72)* with representative examples of the raw data published as Supplemental material. A typical dPCR analysis pipeline involves 1) classification of partitions as positive or negative; 2) determining λ using the proportion of negative (or positive) partitions based on Poisson statistics (Equation 1 below); 3) processing of replicate reactions; and 4) normalization and/or rescaling. Throughout the analysis, it is important to correctly propagate errors to report the uncertainty of the final values *(58)* and to account for system specific technical factors that may influence accuracy *(72)*.

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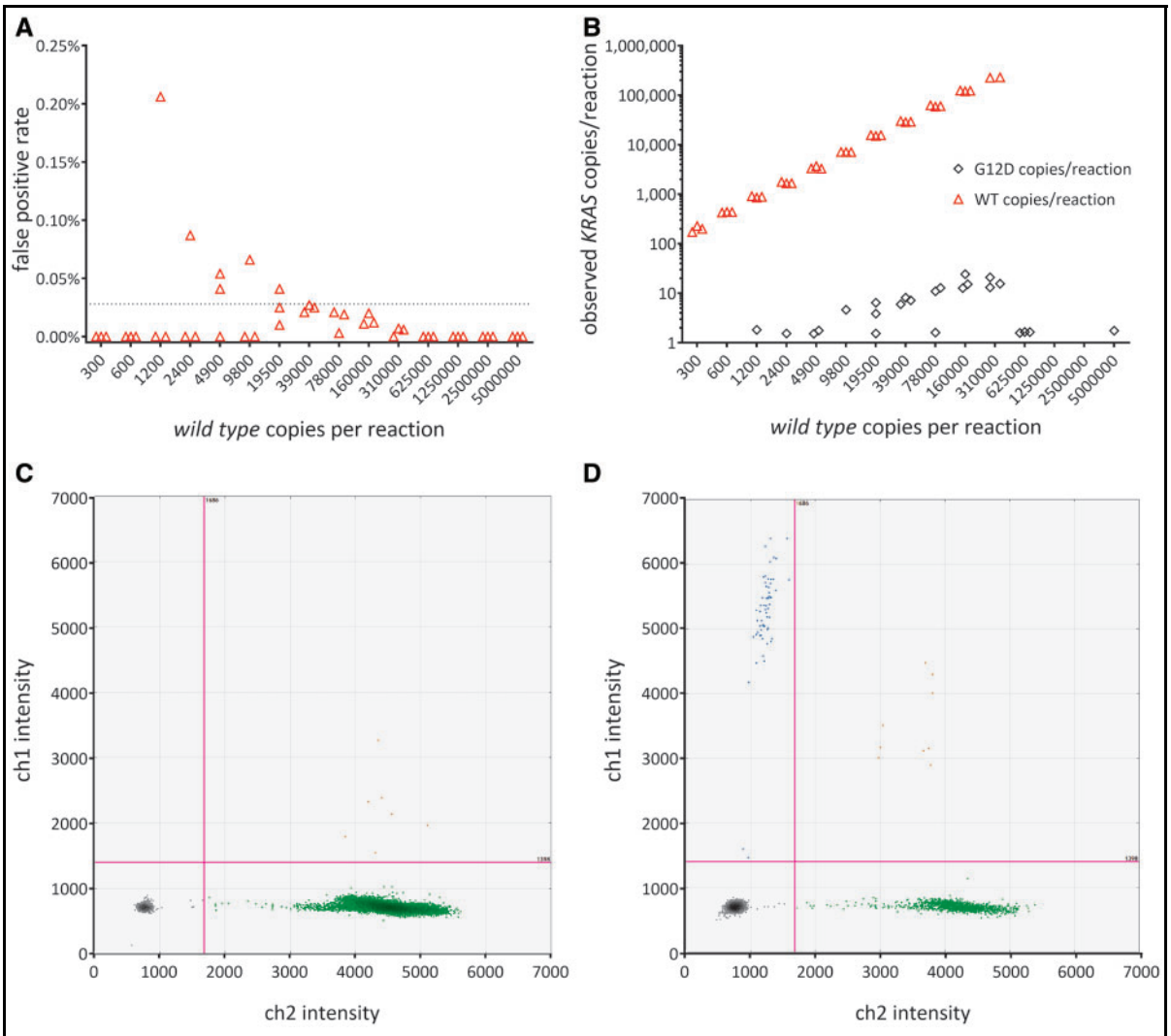


Fig. 4. An example of false positive rate of a single nucleotide variant assay. Example of the *KRAS* G12D mutation using the PrimePCR dPCR assay (Bio-Rad) and Supermix for probes (no dUTP) (Bio-Rad) using the QX200 (Bio-Rad) as described in (*38*). All reactions were performed in triplicate within a single experiment. (A) A 15-point two-fold serial dilution series from ~300 to ~5 million copies of the linearized *KRAS wt* plasmid was analyzed for G12D variant false signal to determine the false positive rate (FPR) over the *wt* dynamic range. FPR is linked to *wt* concentration with too few (<1200) or too many *wt* molecules (>625 000) per reaction reducing the visible FPR. The horizontal dashed line represents the published FPR for this assay (*38*) that is quantifiable between 12 000 to 100 000 *wt* copies per reaction. (B) The corresponding copy number counts for the *KRAS wt* and G12D variant are shown. This demonstrates linearity in the *wt* measurements over the dynamic range (with quantities >60 000 being affected by saturation), and false G12D measurements associated with *wt* concentration. Examples of the two-color plots from the experiment of (C) a single well for the 39 000 *wt* copies/reaction showing the location of the false positive G12D partitions (orange) with the negative (grey) and *wt* only (green) clusters and (D) a positive control reaction using the 5% allelic frequency of the Multiplex I cfDNA Reference Standard Set (Horizon Discovery) to show the location of the single positive G12D cluster (blue). The pink lines represent the threshold to separate the positive and negative partitions and are at the same fluorescence intensity for C and D.

CLASSIFICATION OF THE PARTITIONS

Partitions are classified by setting thresholds that separate the different clusters of partitions based on their fluorescent intensity. If instruments also provide real time PCR plots for each partition, the shape of the sigmoidal amplification curves can also be used to aid classification.

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Each cluster of partitions is assigned as either containing or not-containing target molecules. Multiple partition classification strategies have been developed, including manual and automated procedures with either linear or nonlinear methods (e.g., polygonal gates). Some methods are confined to singleplex assays whilst others can handle multiplex multicolor formats and it may not always be necessary to identify all partition clusters *(59)*.

SETTING MANUAL THRESHOLDS FOR SINGLEPLEX ASSAYS

Manual classification is simple and effective for well-optimized assays that have a clear difference in fluorescence intensity between the positive and negative partitions. However, as complexity associated with the template, assay, and degree of multiplexing increases, manual strategies can be challenging to reproduce and may introduce operator bias. Different batches of probes targeting the same sequence can change the peak resolution (Fig. 2C) and thresholds may need to be redefined. Experiment reports should include the method of manual threshold setting. Analysis by multiple operators is preferred, followed by result comparison to reveal the associated variation. Graphical display of positive and negative controls with their threshold settings should be included in Supplemental material.

AUTOMATED THRESHOLD SETTING

Automated threshold settings can limit bias and increase the robustness of data analysis and reporting. Multiple strategies have been developed by both instrument manufacturers and independent software developers. Each has specific features and assumptions. Statistical strategies include clustering methods *(73–79)*, implementation of extreme value theory *(80)*, and reliance on parametric distributions *(56, 81, 82)*. Automated partition classification methods are only valid if the data fits the assumed distribution *(73, 74, 81, 82)*. Prior statistical analysis (i.e., Kolmogorov-Smirnov) should be performed to help determine the method of choice. Alternatively, ddpcRquant or Umbrella work independently of distribution assumptions *(*78, 80*)*.

RAIN

The ideal dPCR scatter plot has clear space between positive and negative partitions (Fig. 2). However, partitions with intermediate fluorescence, defined as 'rain' are frequently observed (Fig. 5). Rain often results from reduced amplification efficiency that can be caused by several factors, including template accessibility *(83)*, suboptimal annealing temperature *(84)*, PCR inhibitors *(85)*, and mismatches between the assay and the target sequences *(80)*. Studies with probes suggest that longer elongation times, higher cycle numbers, and optimal annealing temperatures may effectively reduce rain *(86)*, and that rain assigned to the positive fraction can improve accuracy (Fig. 5C) *(25, 87–89)*. Hence, these 'rainy' partitions likely contain genuine target sequences and exclusion may lead to a negative bias, especially in cases with trace target, PCR inhibition, or high sequence variation. However, users should investigate rain where it is a problem to establish whether to include it during analysis; there are a variety of methods and tools that can assist in this process *(74, 76–78, 80)*.

CALCULATION OF COPY NUMBER CONCENTRATION

Two assumptions for dPCR to fit the Poisson distribution are that all partitions are of equal volume, and that target molecules are randomly distributed across partitions *(60, 72)*. In practice, this means that each partition has an equal chance of containing target molecules *(90)*. The number of target molecules present within positive partitions may be one, two, or more molecules, and it is currently impossible to determine how many molecules a given positive partition may contain. By contrast, the number of molecules in a negative partition is known. If all partitions are of equal volume, the mean concentration of target molecules per partition (λ) can be estimated from the probability that a partition is negative using the proportion of negative partitions and the Poisson distribution (Eq. 1). This concentration is derived from the number of negative partitions (w) and the total number of partitions in the reaction (n):

$$\lambda = -\ln\left(\frac{w}{n}\right) \quad (1)$$

An equivalent form using the number of positive partitions (k):

$$\lambda = -\ln\left(1 - \frac{k}{n}\right) \quad (2)$$

The simplicity of determining the number of target molecules in this way is one of the strengths of dPCR. Once λ has been calculated, there are a number of downstream calculations that can be made. To calculate the sample concentration (C) of target molecules per unit volume, the most straightforward method uses (Eq. 3), where Vp is the average volume of a partition and D the dilution factor of the sample in the dPCR reaction *(91)*

$$C = \lambda \times \left(\frac{1}{Vp}\right) \times D \quad (3)$$

Equation 3 determines the number of target molecules per unit volume of the sample extract measured by the dPCR. Reporting concentration per sample extract is recommended as it is agnostic to the method used and simplifies comparison.

IMPACT OF PARTITION VOLUME AND VOLUME VARIATION

Current dPCR instrument use predefined partition volumes from the manufacturer that are averaged figures

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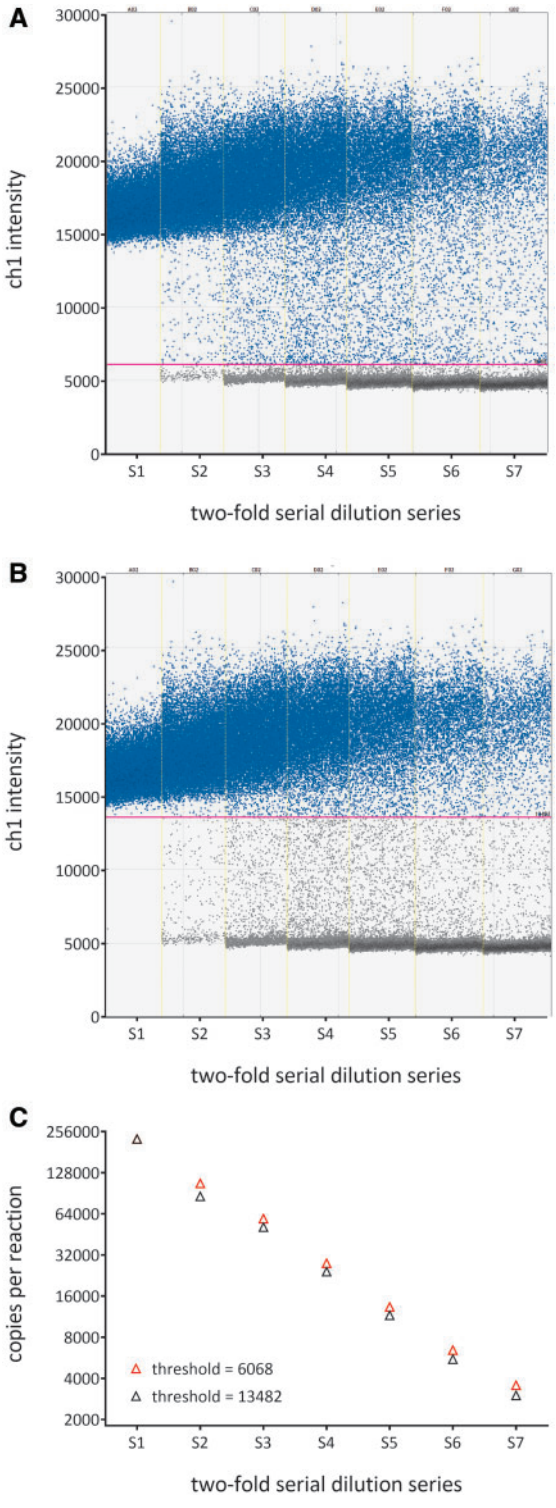


Fig. 5. Inclusion of the rain in the positive fraction of the partitions. (A) A six-point two-fold serial dilution of genomic DNA was prepared.

with no uncertainty value incorporated into the software. Independent validation experiments have revealed that the partition volumes are not constant *(37, 40, 83, 91–93)*. The partition volumes (either assumed or directly measured), and software versions, used should be reported. In addition, volume variance among partitions can result in quantification bias underestimating the target DNA concentration *(60, 72)*. Methods to compensate for partition volume variability are available *(60, 93–95)*. The associated bias is greater at higher concentrations ($\lambda > 2$) *(72)*. A dilution series of template will show a negative bias at higher concentrations if partition volume variance is causing bias *(94)*.

ESTIMATION OF RANDOM ERROR

The binary output of positive and negative partitions follows a binominal distribution, which by definition has a known variance. This theoretical variance in dPCR is often referred to as Poisson error *(96)*. Poisson error is only effective at estimating random error well if the number of positive partitions is sufficiently large and if λ is not extremely low or extremely high *(97)*. Furthermore, Poisson error does not include experimental sources of variance among reactions, such as pipetting errors and sampling variation *(72)*. When errors from multiple sources are combined, the total combined variance should be reported as the uncertainty of the final concentration determination (e.g., using the method from Section 3 in the Supplemental Material 4 from *(58)*). For these reasons, the variation of replicate

Fig. 5. (Continued) dPCR was performed using the QX200 (Bio-Rad) with the Evagreen supermix (Bio-Rad) and 100 nM of each primer that targets a human endogenous retrovirus (HERV-K) target with an amplicon of 80 bp. The annealing temperature was 60 °C and 40 PCR cycles of the recommended Evagreen cycling protocol were performed. The horizontal pink line represents the threshold to separate the positive (blue) and negative (grey) partitions that includes the rain in the positive fraction of the partitions (threshold = 6068; as shown in the figure). (B) The same reactions wells as shown in (A) but with the threshold set high to exclude the rain from the positive fraction of the partitions (threshold = 13482; as shown in the figure). (C) Copy number concentration of the serial dilution either including (red triangles) or excluding (black triangles) the rain in the positive fraction with the threshold number indicated. A systematic bias of 17% resulting in underestimation of the copy numbers was observed when excluding the rain from the copy number calculation.

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experiments used in the analysis should be reported instead of only relying on Poisson error.

EXPERIMENTAL DATA SHARING TO SUPPORT TRANSPARENCY

It is important that experimental analysis can be assessed independently; this is best provided by sharing the raw data so that analyses can be reproduced. This can be done by sharing run files or, to account for the fact that data formats are generated by different instruments, converting the raw dPCR data into Real-Time PCR Data Markup Language (RDML)*(98)*. The latest 1.3 version of RDML, originally developed for qPCR data, now includes dPCR data. The digital raw data are collected in a spreadsheet format and linked to the Extensible Markup Language part of the RDML file that holds the counts for positive and negative partitions allowing reanalysis. Files should be made available either on request or preferably as Supplemental files or on an online repository, such as RDMLdb *(99)*. If data is not available for sharing, authors need to explain why.

The Digital MIQE 2020 Guidelines (dMIQE2020)

The original MIQE and dMIQE guidelines compiled a list of both essential and desirable information for articles applying qPCR or dPCR, respectively. For simplicity this format has been revised, and dMIQE2020 only requires the essential information needed to be included when publishing research using dPCR. For authors, this information can be presented either within the main text or as Supplemental information; a blank dMIQE2020 table is provided for this purpose (Supplemental Table 1). A further update to the table is the inclusion of a new comments section within the checklist for notes and rationale when particular items may not be applicable.

To further support uptake and provide an example of how to complete the table, we have completed the dMIQE2020 table (Supplemental Table 2) for the dPCR experiments performed to generate Figs. 2, 4 and *5* presented in this publication. Supplemental information describing the materials, methods and data used to generate these figures is also provided (online Supplemental Information file).

The dMIQE2020 checklist (Supplemental Table 1) includes the following sections:

SPECIMEN

The specimen is the material taken to be analyzed. Often, only a portion of the specimen, the sample, is analyzed. It is essential that details are provided of what the specimen and sample are and how they were collected; including description of any treatment such as snap freezing or the addition of EDTA to blood. Factors such as volume or mass of the specimen(s) are simple to describe. Sampling procedure, including type of container, handling, and storage conditions, as well as subsampling, must be described because they may result in variation among laboratories. These procedures, and those for nucleic acid extraction (see following section), are being standardized by the European Union funded consortium SPIDIA4P from which European Committee for Standardization (CEN) and International Organization for Standardization (ISO) guidelines are being developed.

NUCLEIC ACID EXTRACTION

Extraction is one of the most crucial preanalytical steps that influences dPCR because it is a major factor in dictating how much nucleic acids are available and present for analysis. Extraction provides two roles prior to molecular analysis. First, nucleic acids are purified, inhibitors removed, and dissolved/eluted in a suitable buffer. Second, nucleic acids can be concentrated, improving analytical sensitivity. It is vital that information about the amount of sample extracted, the amount and type of buffer the resultant nucleic acids were eluted/dissolved in, and any variations applied to the protocol are described. Simply stating that the extraction method was "according to the manufacturer's protocol" is not sufficient.

NUCLEIC ACID ASSESSMENT AND STORAGE

Once the nucleic acid is extracted, it is usually characterized and stored prior to analysis. If initial quantification by total nucleic acid measurement methods (spectrophotometry or calibrated fluorimetry) is performed to guide subsequent template addition, details must be described. Fragment size or differential amplicons *(100)* can be assessed by electrophoresis, and quality metrics may support methodological understanding.

Nucleic acid storage conditions should be reported. A description of temperature, concentration, duration, buffer, and pH, and whether any aliquots were made is essential. While there are many ways to store nucleic acids, stability should be verified when nucleic acids are stored for long (>1 month) periods of time. While DNA can often be directly measured by dPCR after extraction, dilution of concentrated samples may be necessary to prevent dPCR saturation and this should be described if performed. When working with samples of low concentration, material may be lost due to adsorption to container walls, pipette tips, etc. The use of carrier or protective solutions can reduce this effect. Furthermore, repeated freeze-thawing of the sample should be avoided.

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NUCLEIC ACID MODIFICATIONS BEFORE DPCR

The application of methods for modification of extracted templates, such as fragmentation of genomic DNA, must be described. Any preliminary methodological development outlining performance, or associated references, need to be included. DNA modifications, such as bisulfite treatment for assessment of DNA methylation, must be described in detail along with any subsequent dilution and/or purification.

REVERSE TRANSCRIPTION

Along with extraction, the reverse transcription step is a major source of uncertainty and potential bias *(65, 101)*. When conducting reverse transcription, details including the choice of the RT and its concentration, the amount of RNA, incubation temperature and duration, primer concentration, and priming strategy must be described. When conducting two-step RT-dPCR, a detailed description of the separate reverse transcription reaction must be described. Furthermore, when conducting two-step protocols, it is essential to provide a clear description of dilution and/or additional purification steps performed following the reverse transcription step prior to adding the cDNA to the dPCR. Reactions that do not include the RT step (RT negatives) are important to control for DNA contamination that may also be detected and incorrectly measured as cDNA by the RT-dPCR if not defined.

DPCR OLIGONUCLEOTIDES DESIGN AND TARGET INFORMATION

It is essential that information concerning assay design and rationale is provided. Identification of the intended target, including the sequence accession number or official gene symbol, and the location of the amplicon is critical. The specific oligonucleotide sequences are important to include as they are the fundamental components that determine an assays sensitivity and specificity. If these are not available, as with some commercial assays, the amplicon context sequence should be provided *(102)*. Detailed data on assay sensitivity and specificity should also be included. Without this information, validity cannot be ensured by the scientific community.

DPCR PROTOCOL

dPCR can perform measurements with high reproducibility and minimal bias resulting in high accuracy *(38)*. Such characteristics increase the chances that results will be corroborated and of value to the scientific community. Reaction protocol information is essential to include within a manuscript describing results from dPCR experiments. dPCR requires the preparation of prereaction mixtures prior to partitioning and a detailed description of its components, including its volume and the proportion (and total nucleic acid concentration) of template added is needed. It is important to consider the likely final reaction volume (the partition number multiplied by the partition volume) and not the prereaction volume when considering experimental design. This especially important when measuring low concentrations of target as the difference between reaction volume and prereaction volume (termed dead volume) can be large depending on instrument format. Reporting of additional components, such as oligonucleotide concentrations and reaction thermal cycling parameters, are essential for all forms of PCR.

ASSAY VALIDATION

Details of how optimization was performed, such as different temperatures and/or oligonucleotide concentrations used, should be reported, along with pass/fail criteria. It is important to describe analytical specificity and sensitivity and how these parameters were evaluated during experimental validation (for example, choice of control materials). Assessment of analytical specificity varies depending on application and can be influenced by related species (pathogen diagnostics) or endogenous DNA (rare variant detection). Ultimately researchers must be confident that the assay is providing accurate negative and positive results and be cognizant of when this may not be the case.

DATA ANALYSIS

The experimental design should be described, along with the positive and negative controls employed. Examples of positive and negative controls should be included within the publication either in the main body or Supplemental information. When false positive signals are considered acceptable, background examples of these plots should also be included, as well as information outlining how this was considered in the data analysis. Positive and negative controls should serve for assay quality control and threshold setting; they can also be used to identify bias or systematic error in dPCR measurements and preanalytical steps, where suitable materials exist.

Technical replication of biological samples or controls should be described, with the data generated forming the basis of estimation of repeatability and reproducibility, reflecting sources of random variation. Repeatability and reproducibility *(66)* may also be estimated based on experiments performed during assay validation. Replication of the entire experimental process (i.e., replicating samples from the specimen, thus including all preanalytical steps such as extraction and, when measuring cDNA, reverse transcription) is the best estimate for random error of a given measurement.

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Bias or systematic error is more challenging to characterize, but control materials can be used to identify the extent of bias and variation in dPCR measurements and preanalytical steps.

Compiling, normalizing/rescaling, and analyzing the reported dPCR results need to be detailed. The average number and range of the measured partitions is necessary because when this is combined with the partition volume, it provides the actual volume of a given dPCR reaction. dPCR is best initially analyzed using lambda (λ) that can be used to quantify the DNA (or cDNA) concentration of the sample (see Eq. 3). Even when relative amounts, such as fractional abundance are used, λ of the respective targets should also be reported. The ability to report absolute values is unique to dPCR, provides valuable additional information about the experiment and should be included in Supplemental material if not in the main text.

Statistical methods and rationale must be described. To aid transparency and evaluation of conclusions, data should be available either on request or preferably as Supplemental manuscript files or in an online repository, such as RDMLdb *(99)*.

Conclusions

The purpose of the different MIQE publications *(1, 54, 102–108)* is to support authors, editors, and reviewers of manuscripts applying PCR to nucleic acid measurement. The research community can use this as a resource to ensure sufficient detail is published to allow full assessment of the work, while also providing basic guidance on how to approach experimental design, execution, and data-analysis.

The goals of the MIQE series of manuscripts remains three-fold:

1. To enable authors to design, perform, and report experiments that have greater scientific integrity.
2. To facilitate replication of experiments described in published studies where these guidelines are followed.
3. To provide critical information that allows reviewers, editors, and the wider scientific community to measure the technical quality of submitted manuscripts against an established standard.

dMIQE2020 describes optimal reporting of dPCR findings to enhance its scientific impact and maximize the potential of this powerful and unique technology. The ultimate aim is to ensure that published research applying dPCR will be understood from a technical point-of-view and can be reproduced. This in turn supports the validity of associated conclusions and their successful translation for use in applied situations, such as in vitro diagnostic testing.

Disclaimer

Certain commercial equipment, instruments, or materials are identified in this document in order to describe the technology they support. Such identification is neither intended to imply recommendation nor endorsement by the authors nor their affiliated institutions, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Abbreviations: dPCR, digital polymerase chain reaction; qPCR, quantitative polymerase chain reaction; dMIQE, Minimum Information for the Publication of Quantitative dPCR Experiments; MIQE, Minimum Information for the Publication of Quantitative Real-Time PCR Experiments

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

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