



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

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There is growing interest in digital PCR (dPCR) because technological progress makes it a practical and increasingly affordable technology. dPCR allows the precise quantification of nucleic acids, facilitating the measurement of small percentage differences and quantification of rare variants. dPCR may also be more reproducible and less susceptible to inhibition than quantitative real-time PCR (qPCR). Consequently, dPCR has the potential to have a substantial impact on research as well as diagnostic applications. However, as with qPCR, the ability to perform robust meaningful experiments requires careful design and adequate controls. To assist independent evaluation of experimental data, comprehensive disclosure of all relevant experimental details is required. To facilitate this process we present the Minimum Information for Publication of Quantitative Digital PCR Experiments guidelines. This report addresses known requirements for dPCR that have already been identified during this early stage of its development and commercial implementation. Adoption of these guidelines by the scientific community will help to standardize experimental protocols, maximize efficient utilization of resources, and enhance the impact of this promising new technology.

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Digital polymerase chain reaction (dPCR)<sup>14</sup> (1) is progressing from a method that is limited by technical complexity toward a mainstream technology that has unique advantages and applications. It has the poten-

tial to have a major impact on molecular analyses ranging from clinical applications, such as biomarker analysis (2), viral detection (3), prognostic monitoring (4), and fetal screening (5), to research applications such as phage–host interactions (6) and intracellular profiling (7). dPCR can also be applied to assist with the library preparation needed for massively parallel (or next generation) sequencing methods (8).

dPCR involves performing PCR with real-time or end-point data collection in a large number of separate reaction chambers, also termed partitions. Hundreds to millions of these reaction partitions are created with dilutions of template such that at least some of them contain no copies of the target sequence(s) of interest. Results are obtained by counting the number of partitions in which the amplified target sequence is detected (regarded as positive) and the number of partitions in which there is no amplification (regarded as negative). Quantification of the mean number of target sequences per partition is achieved by applying a Poisson correction to the fraction of the positive partitions (9). This compensates for the fact that more than one copy of template may be present in some partitions.

Because the use of 96- or 384-well plates for a single sample is neither practical, affordable, nor very accurate, more widespread implementation of dPCR has required the introduction of nanofluidic techniques and/or emulsion chemistries. Three enhancements associated with dedicated instruments have helped promote the use of dPCR:

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<sup>14</sup> Nonstandard abbreviations: dPCR, digital polymerase chain reaction; qPCR, quantitative real-time PCR; C<sub>q</sub>, quantification cycle; MIQE, Minimum Information for Publication of qPCR Experiments; gDNA, genomic DNA; BLAST, Basic Local Alignment Search Tool; RT-dPCR, reverse transcription digital PCR; gDNA, genomic DNA; RT-qPCR, reverse-transcription qPCR; dMIQE, Minimum Information for the Publication of Digital PCR Experiments.

- Partition volumes have been lowered to as little as 5 pL.
- The partitioning process has been automated.
- The number of partitions has been increased to, in some cases, over 100 000 for a single experiment.

These 3 elements have simplified dPCR and increased its precision while keeping the total reaction volume of a single experiment similar to that of a conventional qPCR.

Currently dPCR instruments achieve partitioning either on chips (10–12) or through water-in-oil emulsions or droplets (13–16):

A. Using chip-based methods, dPCR is performed in small-volume, solid partitions that allow either real-time or end-point analysis of the individual reactions. Representative amplification curves and negative results are shown in Fig. 1. Real-time amplification plots of the individual partitions are not essential for dPCR but are useful during assay development to enable users to understand technical nuances (17). Chips can be used to analyze several samples in parallel and, as with quantitative real time PCR (qPCR), these experiments can be automated. However, the number of partitions that are available when using current chip platforms, typically on the order of a few thousand, is fewer than that offered by the droplet instruments. As a result, the dynamic range of the chips is typically narrower than with droplet instruments. Hence prior knowledge of approximate sample copy number, obtained by performing an initial dPCR titration experiment or qPCR analysis, may be necessary to achieve the desired precision.

B. Emulsion (or droplet) dPCR occurs in partitions made up of water-in-oil emulsion droplets. The emulsion-based instruments allow partition formation as droplets, offering an elegant means of achieving more partitions and lower running costs than most chip-based instruments. Partition number defines the theoretical dynamic range (18) and also has an impact on the quantitative precision at a given concentration. The increased dynamic range of emulsion dPCR allows analysis of a greater range of sample concentrations for any given precision (19). This advantage must be balanced against increased technical complexity and the current need for post-PCR sample manipulation. Current commercial instruments do not include the ability to collect real-time data or perform melting analysis after PCR, but emulsion dPCR with real-time data collection has been performed on laboratory prototypes (13, 20). Commercial instrument data are typically presented as 1- or 2-dimensional scatter plots displaying droplet populations in separate clusters depending on their fluorescence amplitude following singleplex or duplex dPCR (Fig. 2). Although this data format dif-

fers from the more familiar real-time amplification plot, it provides valuable information during assay optimization and validation.

The choice of which is the best instrument is application dependent and requires careful assessment of throughput, budget, and requirements for dynamic range and precision. It is an exciting time for dPCR users, because instrument providers are offering an increasing number of competing formats, each with their respective advantages and disadvantages.

### Applications of dPCR

dPCR offers several key technical advantages over other PCR formats in the following applications.

#### RARE VARIANT MEASUREMENT

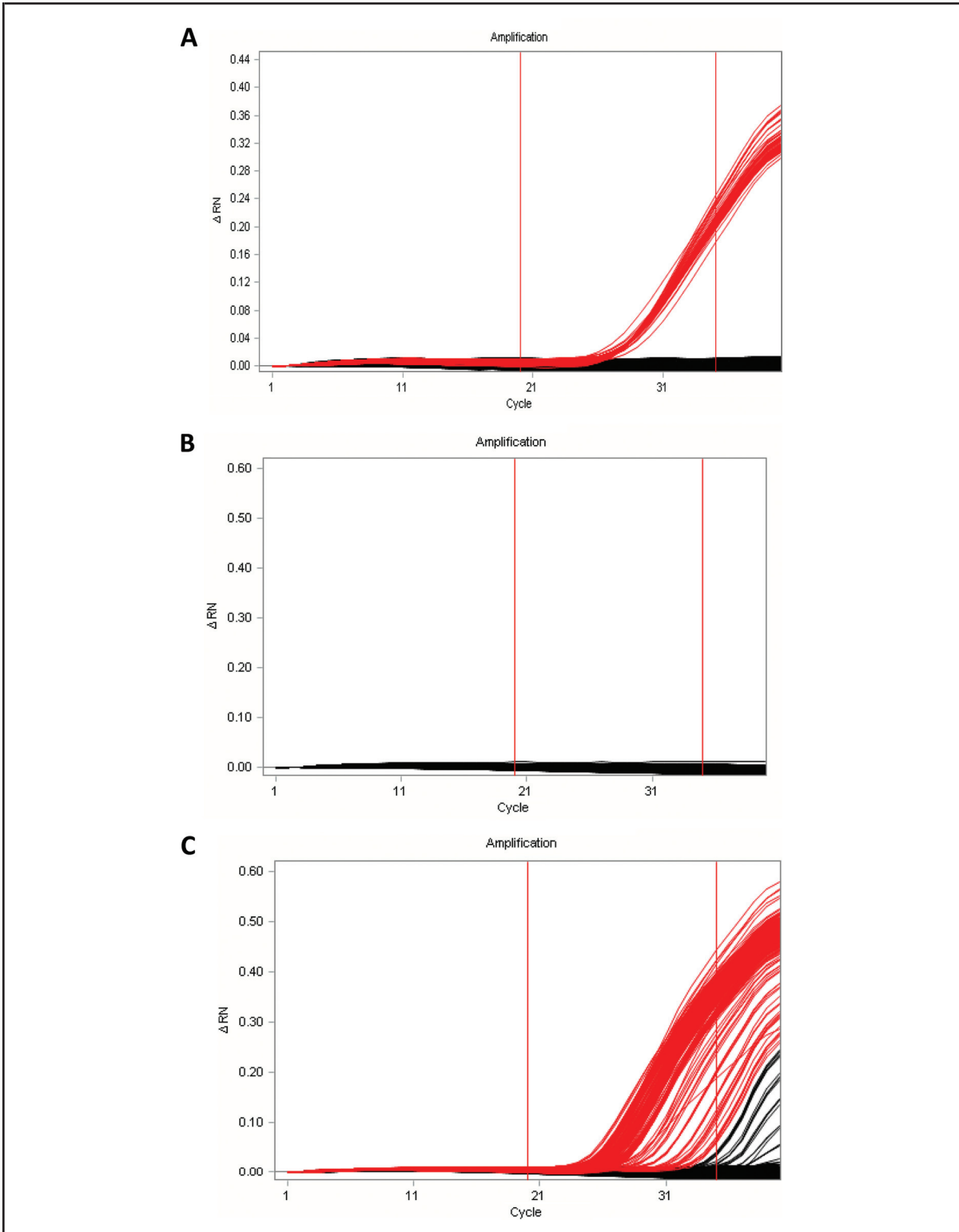
dPCR was initially developed to investigate minority target measurement, for which rare variants are measured in the presence of large numbers of wild-type sequences (1, 21). Detection and quantification of rare mutations can provide a useful tool in several scenarios such as the diagnosis and staging of cancer. By performing a limiting dilution of samples containing rare variants, the background signal that comes from the wild-type genotype is reduced, thereby increasing the likelihood that the rare variant will be detected in any partition that contains it. Variant frequencies as low as 1 in 100 000 have been measured by such methods (18, 22). As the number of partitions increases, so does the sensitivity, providing a cost-effective alternative to rare variant analysis.

#### MOLECULAR COUNTING

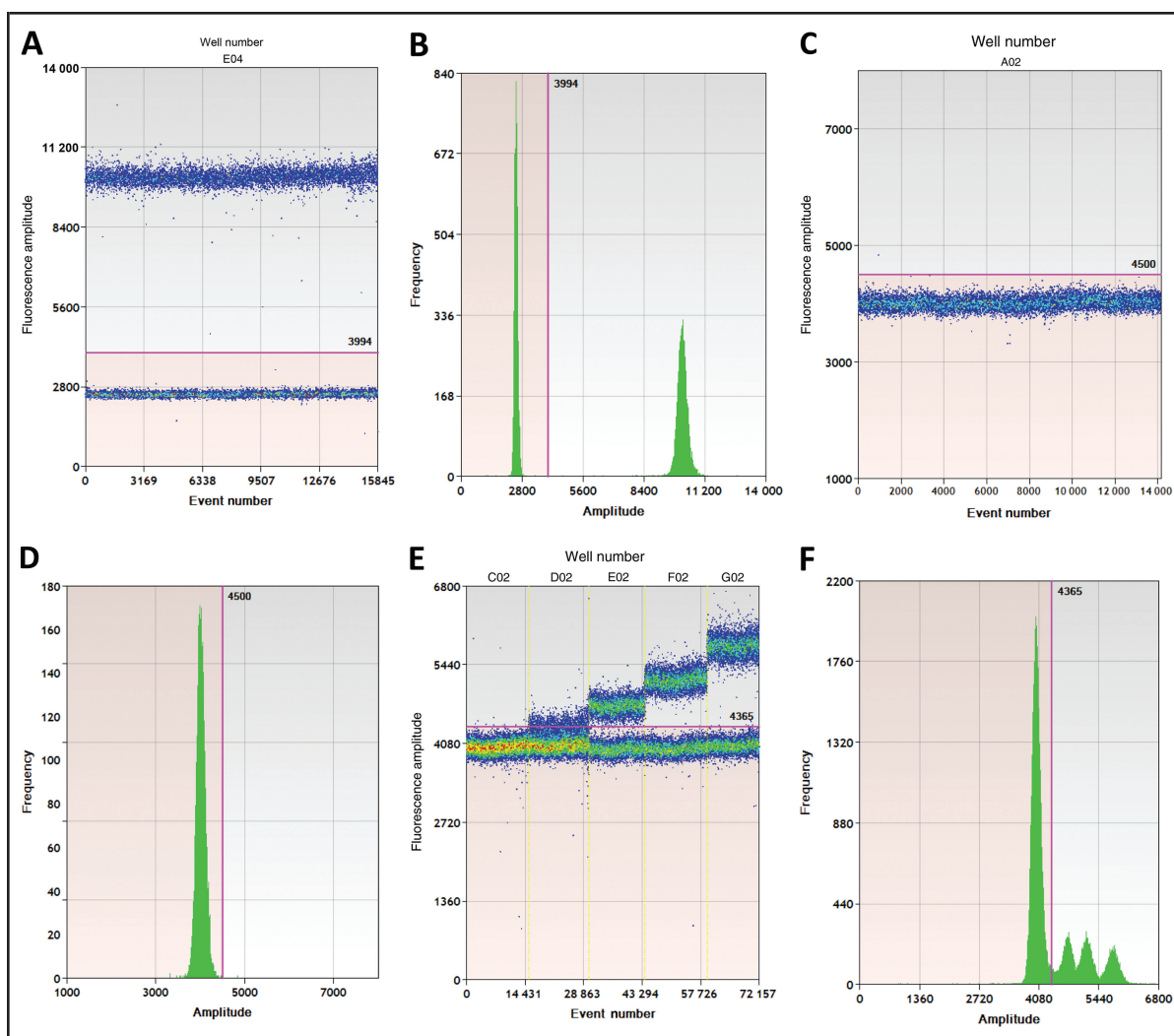
Unlike qPCR, in which the quantification cycle (C<sub>q</sub>) depends on variable features such as the instrument, fluorescent reporter dye, and assay efficiency, dPCR relies on a simple count of the number of successful amplification reactions. The counting of positive partitions in an ideal dPCR is definitive and does not require a calibration curve to convert C<sub>q</sub> to copy number; knowing the partition number and volume is sufficient. This, in theory, makes dPCR more repeatable (by the same operator, instrument, and conditions over a short period of time) and reproducible (by different operators, instruments, and/or conditions) (23) than qPCR. Although repeatability is essential, it is important for a technique to be reproducible as well.

#### APPLICATIONS REQUIRING HIGHER PRECISION

Properly designed and executed dPCR experiments can be more precise than qPCR experiments (24). Such precise measurements are frequently needed when measuring cDNA (complementary DNA generated from RNA) concentrations or estimating genetic copy number variations associated with chro-



**Fig. 1.** Examples of data output from a chip-based real time dPCR instrument (Fluidigm Biomark). Real-time PCR amplification plots showing: (A), a well-optimized assay with clear distinction between positive and negative partitions; (B), no-template control reactions; and (C) experiment with poor demarcation between positive and negative partitions.



**Fig. 2.** Examples of data output from a droplet dPCR instrument (Bio-Rad QX100).

One-dimensional scatter plots and histograms showing an optimized assay with a clear distinction between positive and negative partitions (A and B); no-template control reactions (C and D), and temperature optimization showing incremental separation between positive and negative partitions from wells C02 to G02 (E and F), with poor demarcation in C02 and D02.

mosomal rearrangements or gene/chromosomal dosage (5). qPCR is frequently used to quantify small differences. However, this is often inappropriate because qPCR can, at best and under ideal conditions, measure 1.25- to 1.5-fold differences (25). When compared to qPCR, dPCR is able to measure smaller fold differences of  $<1.2$  (24).

dPCR is a valuable tool for routine molecular measurements that require precision in quantification. However, for this to occur in a timely fashion and with maximal impact, it is important (a) that early research is performed with good dPCR experimental design and (b) that ensuing publications provide adequate experimental

detail. There are dogmas already associated with dPCR that need to be examined and either validated or abandoned. Consequently it is useful to identify which parameters detailed by the original Minimum Information for Publication of qPCR Experiments (MIQE) guidelines (26) apply to dPCR and what additional, digital PCR-specific information should be provided.

### Characteristics of dPCR

#### ABSOLUTE QUANTIFICATION

In qPCR, the term absolute quantification is used to describe measurements that estimate the abundance

of a target relative to that of a standard curve derived from a standard target of defined amount estimated by independent means, e.g., a synthetic oligonucleotide. In contrast, the term “absolute quantification” used in dPCR (8, 14, 27) refers to an estimate derived from the count of the proportion of positive partitions relative to the total number of partitions and their known volume. When the sample is sufficiently dilute, most partitions will not contain template and those that do are most likely to contain single molecules. As the sample becomes more concentrated, the chance of more than 1 molecule being present within a positive partition increases. This does not pose too great a challenge, because the distribution of molecules throughout the partitions approximates a Poisson distribution and a Poisson correction is applied (24, 28, 29). The dynamic range of a dPCR assay can extend beyond the number of partitions analyzed (30) but the assay precision deteriorates at each end. In contrast, qPCR precision deteriorates only at low copy numbers.

Although precise quantification without the need for a calibration curve offers a major advantage, additional factors affect dPCR accuracy. dPCR measures only copies that amplify, and sequence damage, assay inhibition, or poor sensitivity may preclude amplification. Molecular dropout has been demonstrated by observing when partitions containing 2 linked assays amplify only 1 target using duplex dPCR (31). The prevalence of single assay dropout provides an estimate of the templates that have not amplified, which in turn can be used to better estimate the true amount of template (32). Substantial molecular dropout requires a calibration control when absolute counts are required (see Calibrators below). Additional factors that affect template amplification include DNA integrity (e.g. fragment length), chemical modifications (e.g. formalin cross-linking), and denaturation state (single vs double stranded). When measuring RNA the efficiency of the reverse transcription must be considered.

### PRECISION

Precision is a measure of the closeness of agreement between replicate measurements and is usually expressed numerically as SD, variance, or CV under the specified conditions of measurement (23). dPCR can be more precise than qPCR and this precision is applicable at very low copy numbers (17). The ability to measure extremely low concentrations of specific DNA sequences, independent of a standard curve, with high precision, in a complex background, is unique to dPCR. However, there is a need for caution when expounding this advantage of dPCR, because dPCR is currently less versatile than qPCR. qPCR allows fairly

precise measurement over a dynamic range that can exceed 9 orders of magnitude. This is far greater than that offered by the currently available dPCR instruments that are limited by the number of partitions available. Furthermore, for much of this dynamic range the relative SD of qPCR is similar (noise is homoscedastic on the log scale). Conversely, although dPCR can yield very precise measurements, the absolute precision depends on the mean number of molecules per partition (dictated by the original sample concentration and how it has been prepared) (28–30) and the number of partitions. Precision becomes poor when the mean number of molecules per partition is very low and as the number of positive partitions approaches saturation. Furthermore, dPCR is most precise at an optimal concentration of approximately 1.59 molecules per partition (25). Consequently, for the most precise measurements a prior estimate of concentration is required.

The theoretical precision of dPCR appears easier to predict than that for qPCR, because the underlying binomial distribution has known variance, which can be used to compute the CI (29, 33). Early evidence suggests that precision is also more constant between different primer pairs in dPCR than qPCR, making power calculations considerably easier (24).

It is important to note that whereas the technique of dPCR may be both precise and reproducible when measuring a given nucleic acid molecule, accuracy may be affected by the need for extraction from a complex mix of biological material. It is therefore essential that experimental replication is appropriate to the target that is being measured (34). Consequently, experimental design should include sufficient biological replicates to allow replication of the whole experimental procedure. Replication that includes the extraction process will most likely lead to an increase in the associated error of a given dPCR measurement (19). Replicating the whole experimental procedure is desirable because it includes the entire process used on the biological sample. Sample extraction not only increases error because of variation in yield, but also includes differences in copurified factors, such as inhibitors, that may adversely influence the downstream PCR and/or reverse transcription reactions.

### REDUCED SUSCEPTIBILITY TO INHIBITORS

dPCR is relatively tolerant to inhibitors compared to qPCR (35). dPCR is not dependent on amplification curves that may be affected by subtle inhibitors as in qPCR. However, dPCR remains susceptible to gross inhibitors that completely inhibit the reaction, which can be both primer and reagent-specific (36). Control experiments (37) should be included to ensure that inhibitors are not causing undetected problems in

dPCR, especially when a negative result is being reported.

#### REACTION OPTIMIZATION

Very few dPCR publications detail any information regarding PCR assay optimization. It is not clear whether this is an oversight or reflects the fact that assays are generally not optimized, the latter becoming less acceptable as the costs of dPCR decrease. However, although signal generation by dPCR may be less dependent on assays being highly efficient than qPCR, the assays must be able to detect single copies and assay efficiency influences analytical sensitivity (38). As dPCR relies on successful amplification from a large number of reactions, each of which contains a single or low numbers of template molecules, dPCR depends upon well-designed, optimized assays.

Design considerations are similar to those for qPCR, including *in silico* primer specificity screens such as the Basic Local Alignment Search Tool (BLAST), avoidance of primer dimers and secondary structure, and consideration of pseudogenes. Although many commercially available qPCR assays are well optimized, it would be prudent for users of these, and of newly designed assays, to perform laboratory-specific optimization. Although one might expect an assay that works well with qPCR to also perform well when using dPCR, this general assumption should be confirmed empirically. Annealing temperature gradients can be used to optimize the distinction between positive and negative reactions (Fig. 2). Increasing the number of cycles may be necessary with more structurally complex templates, e.g., intact plasmid DNA. Conducting a preliminary assessment to estimate copy number, efficiency, linearity of response, and sensitivity will increase the robustness of any dPCR assay.

One simple way to evaluate a dPCR experiment is to employ multiple assays using different primer pairs (in single or multiplex format) targeting the same template and validating the assays against each other. For genomic DNA (gDNA), new assays can be compared to existing, well-characterized assays targeting single copy regions (39). If results from different assays are in good agreement, this provides another level of confidence that the assays are likely to be optimal. However, if the intention is to perform multiplex analysis, the efficacy of multiplex formats should be compared to singleplex reactions (32).

#### Components of dPCR

##### DNA TARGET

dPCR performance may be best with smaller linear DNA molecules because, as with qPCR, circular supercoiled templates can result in reduced sensitivity (17).

Furthermore, fragmentation of larger, complex gDNA may also improve associated measurement (40). When dPCR is used, there is a unique challenge associated with quantifying sequences that may occur as concatamers (repeats of the same sequence that are present on the same molecule); concatameric sequences cannot be measured individually in different partitions. Such sequence arrangements, which occur normally in eukaryotic, bacterial, and viral genomes, are also associated with certain cancer genotypes (41). Although the precision of dPCR may provide a new clinical opportunity for prognostic monitoring of genomic instability (24), when concatameric sequences are evaluated, some strategy for physical separation is necessary for their independent measurement. Pre-amplification (42), controlled fragmentation using restriction enzymes that do not cleave the target region, and sonication offer potential solutions to this problem.

Another important consideration is whether the DNA being analyzed is single or double stranded when being partitioned. A double-stranded DNA molecule can occupy only 1 partition. However, if that molecule is denatured into 2 single strands they could occupy 2 partitions, leading to a 2-fold overestimation if the user assumes they are quantifying double-stranded DNA (31).

##### RNA TARGET

Reverse transcription dPCR (RT-dPCR) is more complex than simply measuring DNA by dPCR because it requires the additional reverse transcription step to convert RNA to complementary DNA (cDNA). Consequently the assumption that DNA measurement by dPCR can be precise, reproducible, and absolute cannot be readily extrapolated to the measurement of RNA. RNA measurement by reverse-transcription qPCR (RT-qPCR) is notoriously variable, depending on experimental design, including RT protocol (34, 43), RNA secondary structure (38, 44), and choice of reagents, with a varying degree of sensitivity dependent on the numbers of RNA molecules being converted to cDNA (43).

In the same way, quantification of cellular RNA or RNA viruses reflects only the number of target cDNA molecules converted from the original RNA. This may or may not give an accurate estimate for the original concentration of the RNA molecules of interest. RT-dPCR may perform well when measuring the relative amounts of the same RNA from different samples. However, assays targeting different parts of the same molecule may well give different results, it is not recommended that cross-assay comparisons are made unless they are calibrated to control for differences in the generation of cDNA.

## CALIBRATORS

dPCR has been described as a system that provides precise measurement without the need for calibration (8). While a qPCR calibration curve is avoided, we would strongly caution against assuming that dPCR assays or instruments do not need calibration. During experimental setup and characterization, the use of templates with defined amounts (concentration or gene ratio) of material provides valuable information on the accuracy of measurements. Use of such materials is a crucial first step in assay development and error analysis (17, 19, 24, 30). The use of defined templates also provides a valuable tool for interlaboratory comparison. Instrument calibration is particularly important when using dPCR to measure RNA (27, 45), concatameric sequences, and sequences known to be problematic for PCR, such as those with high GC content.

Calibrators need not be in the form of standard curves, as is common with qPCR. Indeed one of the advantages of dPCR is that the control can be a template with a precisely defined quantity, ideally, a DNA or RNA control or reference material containing the target sequence. Calibrators can range from simple lab-generated templates to internationally accepted reference materials (46), depending on the desired level of accuracy.

## CONTROLS

There is currently no way to sequence products from individual partitions to confirm that the correct amplicon is present. Some instruments allow for post-PCR melting curve analysis to support confirmation of product identity. Alternatively, internal probes can be used. When using hydrolysis probes, a compatible dye can be added for additional melting curve analysis in a separate spectral channel of the instrument (47).

Negative controls are crucial for all PCR formats, including dPCR. Negative controls must be used to monitor for false-positive reactions that can result from product carryover from prior reactions or cross-contamination between samples, as well as from non-specific binding and primer dimer formation. Authors should detail negative control information.

In addition to simple negative controls, dPCR requires a threshold to distinguish positive from negative partitions (Fig. 1 and 2). This threshold is used to determine false-positive and false-negative rates, which directly affect assay validity and accuracy. Setting this threshold can be challenging, particularly in instruments that are limited to end-point fluorescence and when the magnitude above background is small. Specific thresholds must be justified when partition results do not clearly separate into negative and positive populations. Appropriate thresholds are particularly important when measuring rare mutations, with probe or

amplitude based (16) multiplex reactions, and when nonspecific double-stranded binding dyes are being used as the reporter. Examples of amplification plots, end-point fluorescence values, or graphic readouts indicating the chosen thresholds should be included in the manuscript or supplemental data.

In assays designed to detect rare variants, a control that contains just the wild type sequence should be included. For example, an experiment measuring a rare mutation in a predominantly wild-type sample should also include a control DNA known not to contain the mutation of interest. Such measurements are further strengthened by the inclusion of controls containing mixes of wild-type and mutant sequences. Where assays are performed in multiplex, clear evidence of demarcation between assays is needed.

## Digital MIQE

The MIQE guidelines were published in 2009 with the specific goal of improving qPCR analyses and ensuring data comparability and reproducibility (26). They were compiled with the aim of encouraging future updates to expand their relevance (48) and here we present some specific considerations for inclusion in publications using dPCR.

A dPCR-specific MIQE checklist, the Minimum Information for the Publication of Digital PCR Experiments (dMIQE), is proposed in Table 1. The additional considerations that are specifically relevant for dPCR are highlighted below. All items are categorized as essential (E) or desirable (D) for dPCR.

### ESSENTIAL INFORMATION FOR dPCR

The following material must be included within a publication or its supplementary information when describing data generated using dPCR.

*Mean copies per partition.* Currently  $\lambda$  (the symbol conventionally used to denote the mean of the Poisson distribution) is used to estimate the mean number of copies per partition. Poisson statistics are necessary to account for the fact that there is a chance that a positive PCR partition contains more than one molecule (29). Current calculations using  $\lambda$  make the assumption that all the partitions are of equal volume. The variation in partition volume of early dPCR instruments systems appears to be small and thus using  $\lambda$  is appropriate (19, 30). However, as more instruments are developed using alternative technology, it will be important that partition volume, and the associated variation, is accurately defined in order to facilitate the use of appropriate methods for estimating the average number of copies per partition. If the estimation of  $\lambda$  is not provided as part of the instrument readout,  $\lambda$  can be calculated

Table 1. dMIQE checklist for authors, reviewers, and editors.<sup>a</sup>

Item to check	Importance	Item to check 2	Importance
Experimental design		dPCR oligonucleotides	
Definition of experimental and control groups.	E	Primer sequences and/or amplicon context sequence. <sup>b</sup>	E
Number within each group.	E	RTPrimerDB (real-time PCR primer and probe database) identification number.	D
Assay carried out by core lab or investigator's lab?	D	Probe sequences. <sup>b</sup>	D
Power analysis.	D	Location and identity of any modifications.	E
Sample		Manufacturer of oligonucleotides.	D
Description.	E	Purification method.	D
Volume or mass of sample processed.	E	dPCR protocol	
Microdissection or macrodissection.	E	Complete reaction conditions.	E
Processing procedure.	E	Reaction volume and amount of RNA/cDNA/DNA.	E
If frozen—how and how quickly?	E	Primer, (probe), Mg <sup>++</sup> and dNTP concentrations.	E
If fixed—with what, how quickly?	E	Polymerase identity and concentration.	E
Sample storage conditions and duration (especially for formalin-fixed, paraffin-embedded samples).	E	Buffer/kit catalogue no. and manufacturer.	E
Nucleic acid extraction		Exact chemical constitution of the buffer.	D
Quantification—instrument/method.	E	Additives (SYBR green I, DMSO, etc.).	E
Storage conditions: temperature, concentration, duration, buffer.	E	Plates/tubes Catalogue No and manufacturer.	D
DNA or RNA quantification	E	Complete thermocycling parameters.	E
Quality/integrity, instrument/method, e.g. RNA integrity/R quality index and trace or 3':5'.	E	Reaction setup.	D
Template structural information.	E	Gravimetric or volumetric dilutions (manual/robotic).	D
Template modification (digestion, sonication, preamplification, etc.).	E	Total PCR reaction volume prepared.	D
Template treatment (initial heating or chemical denaturation).	E	Partition number.	E
Inhibition dilution or spike.	E	Individual partition volume.	E
DNA contamination assessment of RNA sample.	E	Total volume of the partitions measured (effective reaction size).	E
Details of DNase treatment where performed.	E	Partition volume variance/SD.	D
Manufacturer of reagents used and catalogue number	D	Comprehensive details and appropriate use of controls.	E
Storage of nucleic acid: temperature, concentration, duration, buffer.	E	Manufacturer of dPCR instrument.	E
RT (if necessary)		dPCR validation	
cDNA priming method + concentration.	E	Optimization data for the assay.	D
One- or 2-step protocol.	E	Specificity (when measuring rare mutations, pathogen sequences etc.).	E
Amount of RNA used per reaction.	E	Limit of detection of calibration control.	D
Detailed reaction components and conditions.	E	If multiplexing, comparison with singleplex assays.	E
RT efficiency.	D	Data analysis	
Estimated copies measured with and without addition of RT. <sup>b</sup>	D	Mean copies per partition ( $\lambda$ or equivalent).	E
Manufacturer of reagents used and catalogue number.	D	dPCR analysis program (source, version).	E
Reaction volume (for 2-step RT reaction).	D	Outlier identification and disposition.	E
Storage of cDNA: temperature, concentration, duration, buffer.	D	Results of no-template controls.	E
dPCR target information		Examples of positive(s) and negative experimental results as supplemental data.	E
Sequence accession number.	E	Where appropriate, justification of number and choice of reference genes.	E
Amplicon location.	D	Where appropriate, description of normalization method.	E
Amplicon length.	E	Number and concordance of biological replicates.	D
In silico specificity screen (BLAST, etc.).	E	Number and stage (RT or dPCR) of technical replicates.	E
Pseudogenes, retropseudogenes or other homologs?	D	Repeatability (intraassay variation).	E
Sequence alignment.	D	Reproducibility (interassay/user/lab etc. variation).	D
Secondary structure analysis of amplicon and GC content.	D	Experimental variance or CI. <sup>d</sup>	E
Location of each primer by exon or intron (if applicable).	E	Statistical methods used for analysis.	E
Where appropriate, which splice variants are targeted?	E	Data submission using RDML (Real-time PCR Data Markup Language).	D

<sup>a</sup> All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if possible.

<sup>b</sup> Disclosure of the primer and probe sequence is highly desirable and strongly encouraged. However, since not all commercial predesigned assay vendors provide this information, when it is not available assay context sequences must be submitted [Bustin et al. (48)].

<sup>c</sup> Assessing the absence of DNA using a no-RT assay (or where RT has been inactivated) is essential when first extracting RNA. Once the sample has been validated as DNA free, inclusion of a no-RT control is desirable, but no longer essential.

<sup>d</sup> When single dPCR experiments are performed, the variation due to counting error alone should be calculated from the binomial (or suitable equivalent) distribution.



using the number of partitions ( $n$ ) and either the estimated copy number in the total volume of all partitions ( $m$ ) or the number of positive partitions counted ( $k$ ) with the following equations:

Using estimated copies:

$$\lambda = m/n;$$

Using positive partitions:

$$\lambda = -\ln(1 - k/n).$$

Here we propose a new set of symbols to facilitate a generic approach that can be applied to all platforms; i.e., whether the partitions are droplets or chambers. We use  $k$  and  $n$  because the digital distribution is binomial, conventionally considered as  $k$  successes out of  $n$  trials. In this case, a “trial” is the response of a single partition (positive or negative). We propose that reporting  $\lambda$  be considered essential because this and the partition number (see below) constitute the factors that determine the precision of the estimation (25).

**Partition number.** The number of partitions must be reported because the precision of a given result depends on the number of partitions measured. This is particularly important for the droplet-based instruments because the number of partitions varies from run to run.

**Template structural information.** As previously described, there is mounting evidence that template structure and complexity can have an impact on dPCR accuracy. Consequently it is essential that as much information concerning template type (e.g. gDNA/plasmid/virus/cDNA), source (e.g. organism, tissue, cell), and method of processing (e.g. enzyme treated, sonicated, preamplified) is included. Additionally, information on prior manipulation of template with heat and or other denaturing agents should be included. When restriction enzyme digestion is performed, verification is required to demonstrate that the restriction site is not present within the target amplicon.

**Individual partition volume.** An accurate estimation of partition volume is important because an error in the partition volume will produce a bias in estimation of template concentration per unit volume. Different instruments/chips have different partition volumes and this may not be clear to individuals who are not familiar with dPCR or the particular instrument in question.

**Total volume of the partitions measured (effective reaction size).** The sum of the partitions multiplied by the partition volume will enable the total volume of the reaction to be calculated. This is particularly important for droplet-based instruments because the total volume analyzed can vary between different runs because

of interrun differences in the number of partitions generated.

**Comprehensive details and appropriate use of controls.** Controls are important in all PCR analyses and they are even more important in dPCR, for which confirmation of the amplicon identity is either not possible or difficult. Controls are particularly relevant when performing rare mutation analyses.

**Examples of positive and negative experimental results as supplemental data.** Representative amplification plots or end-point fluorescence values must be shown that distinguish positive and negative partitions with clear demarcation of different assays when multiplexing.

**Experimental variance or CI.** Multiple biological replicates are encouraged to assess total experimental variation. When single dPCR experiments are performed, a minimal estimate of variance due to counting error alone must be calculated from the binomial (or suitable equivalent) distribution.

#### DESIRABLE INFORMATION

Desirable information is that which aids a fellow scientist in understanding a study and should be included when it is available.

**Partition volume variance/SD.** This is particularly important when performing absolute quantification. Such basic information should be made available by the instrument manufacturer if not measured by the laboratory. This metric will become increasingly important as more instruments become available.

**Optimization data for the assay, e.g., using temperature gradients, ensuring PCR cycles are sufficient.** When running costs preclude optimization using dPCR, qPCR should be used and PCR efficiency and limit of quantification can be supplied.

**Total PCR reaction volume prepared.** This is included to reflect the fact that many of the instruments require preparation of a much larger initial sample volume than is actually analyzed.

**Limit of detection of calibration control.** Calibrators are particularly important for RNA measurement.

The necessity of quality-assurance measures for dPCR is as urgent as that for qPCR and RT-qPCR. As with qPCR, the major contribution afforded by dPCR over more conventional molecular methods is the potential to quantify target nucleic acids precisely. For this to have maximum impact, careful consideration must be given to experimental design and reporting along the lines established for qPCR (26). The majority of the considerations associated with dPCR are shared with qPCR; however, there are

differences. Table 1 provides a checklist for authors who are preparing a report from a study using dPCR. Items deemed essential are necessary to allow reviewers to assess the work and enable other investigators to reproduce it. Items considered desirable are also important and should be included where possible.

As with the qPCR MIQE guidelines, the purpose of the dMIQE recommendations is 3-fold:

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

The rapid and universal adoption of the dMIQE guidelines should result in more reproducible data and reliable scientific reporting that will increase the impact of the associated research and maximize the contributions of this promising and rapidly developing technology.

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