**Title:** Digital PCR for the Characterization of Reference Materials

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**Abstract:** Well-characterized reference materials support harmonization and accuracy when conducting nucleic acid-based tests (such as qPCR); digital PCR (dPCR) can measure the absolute concentration of a specific nucleic acid sequence in a background of non-target sequences, making it ideal for the characterization of nucleic acid-based reference materials. National Metrology Institutes are increasingly using dPCR to characterize and certify their reference materials, as it offers several advantages over indirect methods, such as UV-spectroscopy. While dPCR is gaining widespread adoption, it requires optimization and has certain limitations and considerations that users should be aware of when characterizing reference materials. This review highlights the technical considerations of dPCR, as well as its role when developing and characterizing nucleic acid-based reference materials.

# 1. Introduction

## 1.1 How digital PCR works

Digital PCR (dPCR) operates using essentially the same chemistry and thermal cycling parameters as quantitative PCR (qPCR), amplifying a specific nucleic acid sequence with primers and measuring the amplification of this sequence with either fluorescently labeled specific probes or a non-specific double-stranded DNA binding dye. However, in dPCR the reaction is fractionated into hundreds to tens of thousands of low volume partitions. These partitions can be either droplets within an oil emulsion or fixed chambers within a cartridge. In both cases, the limiting dilution will result in some partitions containing a target molecule and some partitions not containing a target molecule. If the partition volume is known, the original starting concentration of the material can be calculated by counting the number of positive and negative partitions (See Equation 1). This process relies on Poisson statistics to correct for the probability that a positive partition contains more than one target molecule.

$$Concentration = \frac{\lambda}{Partition\ Volume}$$ ; where $$\lambda = -ln\left(\frac{Negative\ Partitions}{Total\ Partitions}\right)$$

Equation 1.

Unlike qPCR, dPCR does not require an external calibrant for absolute quantification. At its most basic level, dPCR uses an end-point measurement – meaning that the number of positive and negative partitions are counted after all PCR cycles are complete. However, there are also dPCR instruments on the market that offer real-time dPCR. These instruments measure the fluorescence of a partition after each cycle, in each partition, and use that information to create an amplification curve for each partition.

## 1.2 Brief history of digital PCR

dPCR actually predates qPCR; in the late 1980s and early 1990s, several different groups of scientists used what they termed “limiting dilution PCR” and “single molecule PCR” (Morley, 2014), while qPCR was first described as “kinetic PCR analysis” in 1993 (Russel et al., 1993). In 1999, Bert Vogelstein and Kenneth Kinzler coined the term “digital PCR” (Vogelstein and Kinzler, 1999). The first commercial dPCR instrument, the BiomarkHD (Fluidigm), was released seven years later in 2006. Since that time, additional manufacturers have started to produce dPCR instruments (Tan et al., 2023). Over the intervening years, dPCR has become easier to use and more affordable, which has led to increasing use of dPCR throughout the biosciences including with infectious disease (Rutsaert et al., 2018; Salipante and Jerome, 2020; Yoo et al., 2021), cancer (Olmedillas-López et al., 2017; Zhu et al., 2016), inherited diseases (Debrand et al., 2015), genetically modified organisms (Milavec et al., 2014), and water quality monitoring (Tiwari et al., 2022). As of today, there are a variety of dPCR instruments on the market with different

options for various parameters, such as the partition type, number of detection channels, dynamic range and partition size (Basu, 2017; Demeke and Dobnik, 2018; Pecoraro et al., 2019; Rutsaert et al., 2018) [Table 1]. Instruments with more detection channels will allow for more targets to be measured simultaneously, which is advantageous for limited samples (such as liquid biopsy); however, multiplexing requires careful optimization to ensure that there is bleed-through between channels and no interference between primer/probe sets (Bogožalec Košir et al., 2023; Vallone and Butler, 2004; Whale et al., 2016). The throughput and dynamic range capabilities also vary between instruments; more partitions allow for greater dynamic range, but instruments with greater partition number often have lower sample throughput, so researchers often have to prioritize when selecting a dPCR instrument. Some of these instruments are only able to use proprietary reagents, while others are compatible with multiple commercially available PCR reagents. In instruments that allow the use of non-proprietary reagents, some labs have found better results with certain mastermixes (Dong et al., 2016; Kline and Duewer, 2017). End-users should carefully consider the best option for their particular use case when deciding which instrument to select. Some groups have compared results from different digital PCR instruments within the same lab (Dong et al., 2015; Pavšič et al., 2016a) and between multiple labs using a variety of instruments(Pavšič et al., 2017; Whale et al., 2018). In 2015, using a certified reference material, Dong et al showed that when biases in partition volume were accounted for, results between the instruments were comparable. In 2017, Pavšič et al performed an interlaboratory study using both whole virus material and extracted DNA; they found that differences between different instruments in the same lab were comparable to differences between the same instrument in different labs.

For further information on digital PCR, readers are encouraged to read previous digital PCR reviews (Bhat and Emslie, 2016; Kuypers and Jerome, 2017; Powell and Babady, 2018) and Digital MIQE Guidelines Update: Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020 (Whale et al., 2020).

## 2. Reference Materials

### 2.1 The importance of reference materials

A reference material is defined as a “material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process or in examination of nominal properties” (Joint Committee for Guides in Metrology, 2012). Reference materials are used to calibrate measurement systems which facilitates quality control and allows users to determine performance characteristics of instruments and measurement processes. While calibration is obviously important for quantitative nucleic acid measurement, such as for determining a viral load measurement, it may also be important when characterizing the performance of methods that are used to detect the presence or absence of a nucleic

acid. Metrics like dynamic range and limit of detection also require accurately characterized reference materials, even if quantitative measurement is not intended. As a result, reference materials help to ensure that results are comparable across labs (Garcia-Casal et al., 2018; Merlini et al., 2010). Additionally, reference materials can be used to verify that intra-laboratory measurements remain consistent over time (Vesper et al., 2007). A certified reference material (CRM) is a specific type of reference material defined as a "reference material characterized by a metrologically valid procedure for one or more specified properties, accompanied by a reference material certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability" (International Organization for Standardization, 2016). The certificate that accompanies the CRM states its certified quantity along with the uncertainty on that value assignment (which may include factors such as batch homogeneity, impurities, etc.). The certificate also generally includes information about the source material for the CRM (such as human, animal, bacterial, etc.) and the intended use of the CRM (such as qPCR).

While CRMs have advantages relative to non-certified reference materials, it is not always possible to create a CRM for biological materials due to material composition (such as a complex biological matrix that requires extraction prior to measurement). In cases where a CRM is impractical, a reference material that does not have the same level of rigor as a CRM can still be used to maintain measurement consistency across laboratories and time points; however, care must be taken when switching suppliers or utilizing a new batch of the reference material. In some cases, a manufacturer will perform bridging studies to harmonize measurements between the previous and current batches of a reference material.

### 2.2 Nucleic acid-based reference materials

Nucleic acid analysis is widely used in clinical diagnostics (Jing et al., 2018), food analysis (Ren et al., 2017), and environmental monitoring (Cao et al., 2015). The number of nucleic acid-based tests and their applications are increasing as the life science sector becomes more established. Consequently, nucleic acid-based reference materials for the development, validation, and calibration of these nucleic acid-based tests are increasingly needed. Reference materials are required for a diverse spectrum of applications, from targeted qualitative examination (e.g., presence/absence of a target) to quantitative analysis (relative or absolute) of specific sequences to non-targeted detection of unknown sequences. For example, to decide on the best course of treatment for cancer patients, physicians often measure the expression level of *HER2 (ERBB2)*, *EGFR*, and/or *MET* relative to other genes. To improve these measurements, the National Institute of Standards and Technology (NIST) developed reference materials SRM 2373 and RM 8366 (NIST, Gaithersburg, USA) using extracted DNA from cell lines with different copy numbers of these genes; the copy number concentrations

of these materials were value assigned based on dPCR measurements (He et al., 2019, 2016). In another example, EURM-019 (JRC, Geel, Belgium), a synthetic partial SARS-CoV-2 RNA value assigned with dPCR, was used to calibrate qPCR measurements of SARS-CoV-2 in wastewater samples (Rector et al., 2023). Reference materials can be of varying complexity from simple synthetic or purified nucleic acids in an aqueous buffered solution (e.g., Tris-EDTA) such as the aforementioned RM 8633, SRM 2373 and EURM-019, to complex matrix materials (e.g., blood or saliva), with the type of reference material required varying according to the particular step in the analytical procedure. Simpler materials (e.g., extracted nucleic acids in buffered solution) are generally used further downstream in the testing process, such as for qPCR calibration curves; while reference materials in more complex matrices (blood, saliva, etc.) are intended to control for the entire procedure, including pre-analytical steps, such as extraction, and downstream steps. For example, another nucleic acid based reference material produced by NIST, SRM 2391d - PCR-Based DNA Profiling Standard (NIST, Gaithersburg, USA), contains a component with cells spotted onto a small piece of paper which requires nucleic acid extraction by the laboratory using the reference material (National Institute of Standards and Technology, 2023; Steffen et al., 2017).

**2.3 Historical measurement of nucleic acid-based reference materials prior to dPCR**

National Metrology Institutes are government entities that are charged with maintaining national measurement standards (Milavec et al., 2022); they do this partly through the distribution of physical reference materials, and they continually try to improve the methods for the characterization of these materials. Prior to the development of instruments that allowed routine dPCR analysis, National Metrology Institutes and other reference material producers typically used indirect methods such as UV spectroscopy (Prokisch et al., 2001; Trapman et al., 2002; Vallone et al., 2013) or nucleic acid binding dyes (Bhat et al., 2010; Holden et al., 2009) to measure and certify the concentration of nucleic acid-based reference materials. Inductively coupled plasma mass spectrometry (ICP/MS) and inductively coupled plasma optical emission spectroscopy (ICP/OES) were also investigated for the measurement of low concentration nucleic acid materials (Holden et al., 2007) prior to the widespread adoption of dPCR. These methods measure the total amount of nucleic acid present in a sample, and while some methods can distinguish between DNA and RNA, indirect methods are typically unable to provide information on sequence identity, fragment size or integrity of the sample. Thus, the total concentration measured may also include any impurities such as degraded nucleic acid, carrier nucleic acid, or other contaminating nucleic acid, if these are present in the sample; which may artificial increase the concentration, relative to what the researcher is trying to measure. In contrast, dPCR measures specific sequences as long as they are accessible and amplifiable (Duewer et al., 2018). These indirect methods are also limited in that only nucleic acid extracts in

aqueous solutions can be directly measured. For example, nucleic acid in serum or plasma would require an extraction process, in which an unknown amount of nucleic acid is lost. Although extracted nucleic acid is also required for most dPCR analyses, in recent years several groups have performed “direct dPCR” where the nucleic acid concentration is measured without a separate extraction step (Pavšič et al., 2016b; Shin et al., 2022).

## 3. Factors Affecting the Accuracy of dPCR Measurements

### 3.1 Overview of dPCR for nucleic acid reference materials

Since it does not require an external calibrator and can measure low concentrations of a target sequence within a complex background, dPCR is replacing indirect methods for nucleic acid-based reference material certification. Unlike indirect methods, dPCR utilizes the same biochemical mechanisms as qPCR, meaning that factors that affect dPCR measurements (e.g., secondary structure, degradation) will also affect qPCR, making dPCR certified reference materials more commutable with clinical specimens measured using qPCR methods. On the other hand, dPCR is distinct from qPCR in several ways. First, the end point analysis provides the copy number concentration of amplifiable target DNA sequence from a single reaction. The concentration measurements from dPCR can be easily applied to downstream processes that require intact nucleic acid (e.g., next generation sequencing, qPCR calibration curves). Also, as the PCR amplification reactions occur within individual partitions which are measured at the end-point, dPCR tends to be more resistant to PCR inhibitors relative to qPCR (Dingle et al., 2013). Despite these advantages, dPCR still requires optimization to obtain reliable measurement results, especially when characterizing and certifying reference materials. If needed, other methods for nucleic acid quantification can be used in parallel with dPCR to ensure its accuracy. dPCR can be used throughout the entire life cycle of the reference material characterization process, starting with the initial suitability testing of the bulk material, and progressing through homogeneity, certification and finally periodic stability testing of the material (Figure 1).

### 3.2 Assay design

Whenever possible, multiple well-validated dPCR assays that span the target sequence should be used to measure the concentration of the material. If only one assay is used, the concentration may be underestimated due to suboptimal assay design or secondary structure of the nucleic acid. Multiple assays yielding the same value also increase confidence that the nucleic acid-based material is sufficiently intact for analysis. In addition, assays should be fully optimized in terms of annealing temperature, primer/probe concentration and cycle number (Huggett et al., 2013). dPCR assays should have a clear separation between the positive and negative partitions, based on the fluorescence signal. These parameters are also applicable for RNA measurement

using reverse transcription digital polymerase chain reaction (RT-dPCR). Additionally, for RT-dPCR, the choice of primers for reverse transcription (RT) can dramatically affect the RT efficiency and in turn the measured copy number value (see section 4.2 for further information on RT-dPCR). In multiplex reactions combining PCR assays for more than one target, all the primer and probe sequences should be validated, both separately and together, to avoid interference (Whale et al., 2016).

### 3.3 Selection of appropriate reference genes for ratio-based materials

Ratio-based measurements are commonly used in cancer testing (Wang et al., 2023), prenatal screenings (Lee et al., 2018), and GMO quantification (Demeke and Dobnik, 2018). dPCR can also be used to measure ratios between multiple targets in a sample. In these types of measurements, the gene or genes selected as the "reference" gene(s) must be thoroughly characterized. Multiple reference genes should be carefully compared. For example, when gene amplification occurs within a genome, such as for copy number variations in cancer, one must ensure that the reference gene copy number is not also altered; otherwise the entire measurement can be skewed. If multiple cell lines are used, the genes must be characterized in each cell line used, to verify the reference gene copy number. For example, during the certification of a *HER2* gene copy number certified reference material (SRM 2373, NIST, Gaithersburg, MD), the National Institute of Standards and Technology used four unique reference genes to calculate the *HER2* ratios in five different cell lines (He et al., 2016), due to the fact that the "traditional" reference gene for *HER2* measurements, *CEP17*, also varies in its copy number (Wang et al., 2023; Zhu et al., 2016). Another example for ratio-based materials is non-invasive prenatal testing (NIPT), where chromosomal aneuploidy such as Down syndrome (21 Trisomy) is screened by analyzing plasma DNA from the maternal blood. For accurate NIPT results, precise DNA quantification in the ratio (affected chromosome vs reference chromosome) is required. When dPCR is used for NIPT, the choice of reference gene(s) becomes critical, because at most 10 % of maternal plasma DNA originates from the fetus. Accurate measurements for the reference gene(s), therefore, are a critical component of the measurement process in ratio-based materials.

### 3.4 Partition volume

The partition volume is required to calculate the starting concentration of the material (See Equation 1). If the partition volume is overestimated, the starting concentration will be underestimated. Likewise, if the partition volume is underestimated, the starting concentration will be overestimated. Manufacturers of dPCR equipment provide a nominal partition volume but depending upon the technology the partition volume may differ slightly between lots of materials (master mixes, cartridges, etc.) (Corbisier et al., 2015; Košir et al., 2017). For most use cases, the actual partition volume is sufficiently close to the provided partition volume. However, in an effort to obtain SI traceable measurements, some National Metrology Institutes measure the dPCR partition volume

(using a specific lot of reagents) as part of the certification measurement process for their reference materials. Most of this work has been done in droplet based dPCR systems (Dagata et al., 2016; Emslie et al., 2019; Košir et al., 2017), though several groups have also attempted to measure the partition volume in chamber based dPCR systems (Bhat et al., 2009; Dong et al., 2015). In some dPCR platforms, this type of user measurement is simply infeasible, leaving users with no other option than relying on the value provided by the manufacturer. When possible, the partition volume uncertainty should be accounted for in the measurement uncertainty of the target concentration for value assignment of reference materials and also in routine measurements.

**3.5 Subsampling error for rare event quantification**

Because dPCR can detect very specific nucleic acid sequences in a background of other, non-target nucleic acid sequences, it is often used for the detection of very rare variants in samples that are predominated by another variant, such as for cancer variant monitoring and NIPT measurements. In these instances, users should be aware of subsampling errors, which may occur depending upon the amount of sample analyzed. Subsampling error can occur when only a portion of the larger sample is tested, especially when the targets are at a very low concentration. The subsampling process introduces an unavoidable source of error, which is not specific to dPCR. When there are rare events within the original sample to detect, the distribution of the results is usually more like a Poisson distribution than a Gaussian distribution. The subsampling statistics are as follows (Equation 2), the standard deviation ($\boldsymbol{\sigma}_m$) of the targets per subsample is calculated as square root of $m$ , where $m$ is the expected number of targets in the subsample (Basu, 2017). The normalized measurement uncertainty due to subsampling ($u_s$) is given below. For a 95% confidence interval $z_c$ should be 1.96.

$$u_s = z_c \frac{\sigma_m}{m} = z_c \frac{\sqrt{m}}{m} = (1.96) \frac{\sqrt{m}}{m}$$

Equation 2.

For rare events quantification, the distribution of the targets, such as the mean, will have relatively large uncertainties.

**3.6 Preparation of the nucleic acid material**

When producing nucleic-acid based reference materials that will be characterized with dPCR, the secondary structure and integrity of the nucleic acid must be considered, both in terms of accurately value-assigning the material and in making the material similar to the samples intended to be analyzed. The secondary structure of the nucleic acid (e.g., supercoiling) can negatively affect the quantification (Dong et al., 2015, 2016; Kline and Duewer, 2017; Yoo et al., 2016). In general, circular DNA materials (such as plasmids)

should be linearized before dPCR measurement in order to be accurately quantified (Dong et al., 2016). However, note that if the reference material is linearized and the test samples are not linearized, the test samples may appear artificially low when the linearized reference material is used to calibrate the sample. Therefore, there is value in creating a reference material that is as similar to the test sample as possible.
Under some circumstances, DNA fragmentation may be necessary before partitioning to improve the accuracy of the measurement (Pecoraro et al., 2019); this would apply to both the reference materials and the test samples. One dPCR manufacturer recommends enzyme digestion for genomic DNA input greater than 66 ng per 20 µL reaction to decrease the viscosity of the solution (Kaihara et al., 2016). Enzyme digestion may also be necessary to separate tandem gene copies in copy number measurements, so that multiple copies will segregate to separate partitions. Fragmentation of the DNA by sonication has been reported to improve the accuracy of measurement in analyzing mitochondrial DNA (Vitomirov et al., 2017). However, fragmentation of DNA is not always required and should be considered on a case-by-case basis (Devonshire et al., 2015; Jacchia et al., 2018).

### 3.7 Other preanalytical factors

Some DNA extraction methods may impact the dPCR quantification. Even though dPCR is generally less prone to inhibition than qPCR, the presence of enzymatic inhibitors, which can confound and undermine downstream analyses, should be evaluated in the process of implementing a new dPCR assay or new sample types. In addition, some reagents in the extracted DNA solution may interfere with the initial partitioning (such as droplet generation). Moreover, whether the DNA subjected to analysis is double-stranded DNA (dsDNA) or (partially) single-stranded DNA (ssDNA), and under which circumstances the dsDNA might separate into ssDNA, also needs to be considered during the DNA extraction process, sample transportation, and long-term storage (Vallone et al., 2013).

### 3.8 Additional Sources of Uncertainty with dPCR measurements

In addition to partition volume, assay design, gene target selection, subsampling and pre-treatment of the nucleic acid materials, there are a few other factors which contribute to dPCR uncertainty. One such factor is such as partition classification (e.g., “rain”), which becomes more complicated as the number of detection channels increases (Vynck et al., 2023). Although some groups have published data indicating that “rain” is typically composed of late starting positives (Kline and Duewer, 2017), manual threshold setting can be subjective, resulting in different measured concentration values. To minimize this, some researchers have developed automated programs to help set thresholds (Jones et al., 2014; Trypsteen et al., 2015). The Poisson distribution also adds uncertainty to the dPCR concentration, though this is mainly significant at extremely low concentrations (very few positive partitions) or at extremely

high concentrations (very few negative partitions) (Basu, 2017; Whale et al., 2020). Additionally, when dPCR is used to measure a single nucleotide variant in a highly concentrated wild type background, the false positive rate must be take into consideration (Whale et al., 2020, 2018). For further information on designing optimal dPCR experiments, readers should consult the Digital MIQE Guidelines Update: Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020 (Whale et al., 2020).

4. **Limitations of dPCR for reference material characterization**

**4.1 Materials requiring extraction are more difficult to fully characterize**

While dPCR can be made SI-traceable for purified DNA and RNA solutions, it is more challenging to fully characterize materials that require nucleic acid extraction. Nucleic acid extraction releases, and often concentrates, DNA and RNA from cells and removes substances that inhibit PCR (such as plasma, calcium ions, fats, polysaccharides) (Schrader et al., 2012). Extraction methods can vary greatly in their efficiency in extracting nucleic acids from biological samples and removing PCR inhibitors (Claassen et al., 2013; Devonshire et al., 2014) which can lead to bias impacting the subsequent dPCR step (Devonshire et al., 2015). While samples can undergo "direct dPCR" (which typically involves only a heat lysis step), PCR inhibitors can remain in solution and lead to under quantification of the sample (Pavšič et al., 2016b). Nonetheless, reference materials in a complex matrix, such as those mimicking clinical plasma samples, can confer a high-quality standard if the measured value is based on the total DNA amount regardless of the DNA extraction process. Furthermore, dPCR has been shown to provide a useful estimate of the nucleic acids quantities within complex reference materials, which can aid their application when used to evaluate routine tests (Vierbaum et al., 2023, 2022).

**4.2 Uncertainty about reverse transcription efficiency for RNA materials**

When measuring the amount of RNA in a sample, a reverse transcription step is required to convert the RNA into complementary DNA (cDNA) before the PCR. This can be performed either prior to the partitioning or at the same time in a single reaction, referred to as two-step RT-dPCR and one-step RT-dPCR, respectively. Since the sample is partitioned prior to reverse transcription in one-step RT-dPCR, even if the reverse transcription enzyme creates multiple cDNA templates per RNA molecule, it will still only result in one positive partition per RNA template; therefore, one-step RT-dPCR is unlikely to overestimate the copy number concentration. However, due to less than 100% reverse transcription efficiency, one-step RT-dPCR may underestimate the true concentration of RNA. With two-step RT-dPCR, the reverse transcription reaction occurs independently, prior to partitioning. Since the reverse transcription and PCR components are separated, each step can be optimized separately, potentially allowing for a more efficient reverse transcription reaction. However, in two-step RT-dPCR, it is

theoretically possible that the reverse transcription enzyme can create more than one cDNA template per RNA molecule, leading to an overestimation of the true concentration. Some groups have compared one-step RT-dPCR to two-step RT-dPCR with mixed findings as to which yielded higher concentrations (Casmil et al., 2023; Falak et al., 2022; Malla et al., 2023; Niu et al., 2023; Pinheiro-de-Oliveira et al., 2019). To provide metrological traceability, orthogonal methods that utilize distinct measurement principles are often used in parallel with dPCR. These include direct counting and stable isotope dilution mass spectroscopy. In 2022, Niu et al compared one-step RT-dPCR to IDMS and found that RT-dPCR resulted in approximately 10% lower concentration, suggestive of incomplete reverse transcription (Niu et al., 2022). RT-dPCR will require further investigation with orthogonal methods to sort out reverse transcription efficiency and further increase method accuracy.

### 4.3 **Inability to amplify highly fragmented materials**

In liquid biopsy, cell-free DNA (cfDNA) has become a comprehensive biomarker in the fields of non-invasive cancer detection and monitoring, organ transplantation, prenatal genetic testing and pathogen detection. In an amplicon-based assay, the amplifiable fraction (if randomly fragmented) can be approximately calculated using the sum of 1 and the difference of amplicon length (a) and fragment length (f) divided by fragment length (Equation 3).

Equation 3. $$Amplifiable\ Fraction = \frac{(1+f-a)}{f}$$

dPCR amplification efficiency is amplicon length and fragment length dependent. Since cfDNA is fragmented to an average of 165 bp in blood, the detection efficiency of cfDNA is higher when the amplicon size is shorter. Accurate quantification of cfDNA is difficult due to the short fragment length; and it will be even more challenging to quantify highly fragmented ultrashort DNA (≈50 bp), since it is even shorter (Hudecova et al., 2022; Li et al., 2020). dPCR is being used in liquid biopsy to quantify nucleic acids with high sensitivity; it is currently one of the best nucleic acid quantitation choices for highly fragmented materials; however, sample integrity must be factored into the analysis.

### 4.4 **Orthogonal methods to supplement dPCR measurements**

Given these limitations for dPCR measurements, several groups have examined orthogonal methods to measure both DNA and RNA concentrations. One international interlaboratory study compared dPCR results from 10 laboratories to orthogonal methods including isotope-dilution mass spectrometry, capillary electrophoresis and flow cytometric counting (Yoo et al., 2016). Another study using the same plasmid material, specifically examined the effects of supercoiled DNA on dPCR, and compared the results from dPCR to direct flow cytometric counting (Dong et al., 2016). The results

showed that properly optimized dPCR agrees well with orthogonal measurements for DNA materials. RNA materials, in addition to requiring the same optimization steps as DNA materials, also require a reverse-transcription step prior to the dPCR portion. To circumvent the need for reverse transcription, some researchers have demonstrated that RNA molecules can be directly counted, in a sequence specific manner (Yoo et al., 2022). This method is independent not only from reverse transcription but also from PCR amplification; however, these direct counting methods use very specialized, custom equipment, so they are much less accessible than dPCR. Orthogonal methods can also be used to estimate the extraction efficiency. In one study, stable isotope labeled mass spectroscopy was applied to quantify total DNA in plasma/serum samples by using stable isotope labeled DNA as an internal calibrant. Of note, the labeled DNA needs to be carefully verified for the labeling and also to be closely matched to the targeted DNA types (Kwon et al., 2019). Using this approach, SI-traceable DNA measurement can be achieved for DNA in complex biological matrices. The generally close agreement of dPCR with orthogonal methods demonstrates that dPCR is an accurate, reliable quantification method, despite the aforementioned limitations.

**5. Further work analyzing dPCR measurements**

Although dPCR has been shown to be highly reproducible among laboratories (Burke et al., 2023; Whale et al., 2017), several areas, such as those mentioned in Sections 3 and 4, still require additional investigation to improve the accuracy of dPCR. Comparison of dPCR and RT-dPCR to orthogonal methods will be particularly informative with regard to the true accuracy of the technique. As mentioned previously in this work, National Metrology Institutes are government entities responsible for maintaining measurement standards. In addition to distributing physical standards, these institutes often also conduct research into the accuracy and biases of various measurement procedures and they often collaborate with their counterparts in other countries to investigate measurement comparability. The Nucleic Acid Analysis Working Group (NAWG), which is part of the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology is working to resolve issues related to the accuracy of dPCR (Kaarls, 2018; Milavec et al., 2022). The NAWG is composed of National Metrology Institute representatives from over 20 countries which provide DNA and RNA measurement services to their stakeholders. The group conducts interlaboratory studies among its members to elucidate nucleic acid measurement issues and areas of bias, with many of these studies focusing on dPCR (Burke et al., 2023; Whale et al., 2018; Yoo et al., 2016). These large interlaboratory studies, as well as smaller collaborations between institutes, help to ensure that dPCR characterization of reference materials is comparable globally. Further studies on RNA materials using reference materials certified with orthogonal techniques (such as IDMS) will help to elucidate the accuracy of RT-dPCR, particularly with regard to one-step vs two-step techniques.

## 6. Concluding remarks

As nucleic acid analysis tests are increasing every year, reference materials are vital to ensure the reliability and comparability of these types of measurements; dPCR has unique attributes that make it ideal for the characterization of nucleic-acid based reference materials. It does not require a pre-existing calibrator and it is capable of very specific sequence detection in a background of non-target sequences. Additionally, dPCR uses the same PCR-based amplification technique as many of the downstream applications (i.e. qPCR, NGS), meaning that elements that affect dPCR will also affect these downstream applications. However, dPCR assays must be carefully optimized, especially when used for the characterization of reference materials. When characterizing ratio-based materials, it is vital to investigate the copy number of all genes. For rare variant detection, one must also consider subsampling error. Users should also be aware that it is more challenging to fully characterize materials that require nucleic acid extraction.

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Tables & Figures

| Parameter | Available Options |
|---|---|
| Partition Type | Droplet or Chamber |
| Throughput | 1 to 96 |
| Number of Detection Channels | 2 to 6 |
| Number of Partitions Per Reaction | ≈ 1,000 to ≈ 30,0000 |
| Dynamic Range | 2 log to 5 log |
| Reagent Type | Proprietary or Open |
| Partition Size | ≈ 1 nL |
| Measurement Capability | End Point or Real Time |

**Table 1. Digital PCR instruments vary across a number of parameters.** The partition type, throughout, detection channels, number of partitions, dynamic range, reagent type, partition size and measurement capabilities should all be considered when selecting a dPCR instrument.

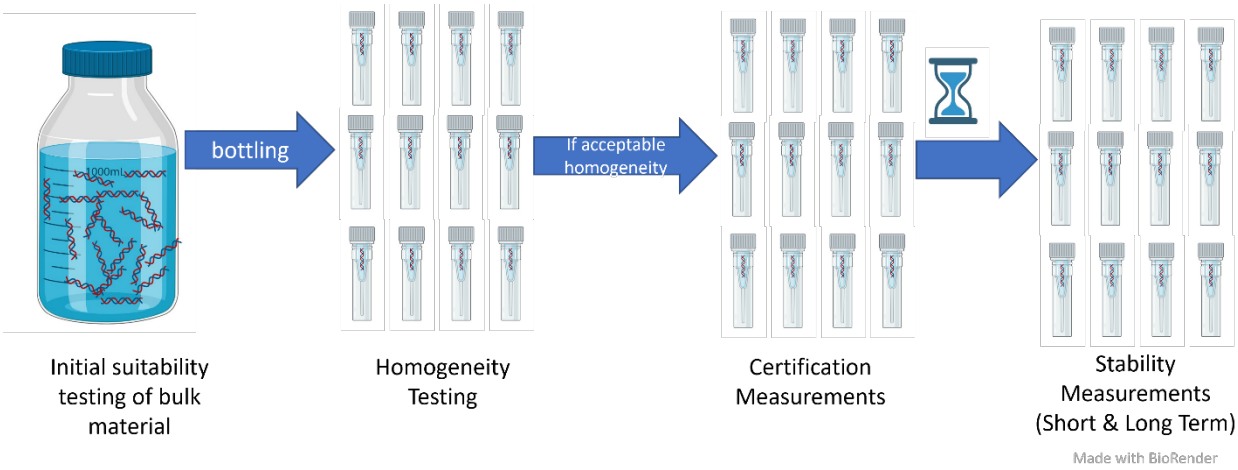


**Figure 1**. **Digital PCR Measurements throughout the reference material process**. dPCR can be used for the initial characterization of the bulk material, to make sure the material will be suitable as a reference material (i.e. sufficiently intact for analysis, purity, etc.). dPCR can also be used for homogeneity testing, to make sure the variation between units of material is acceptable; for this process, typically one or two dPCR assays are used to measure a subset of the units bottled non-consecutively (i.e, every 20$^{th}$ unit or every 100$^{th}$ unit). If the material is confirmed to be sufficiently homogeneous, a subset of units can be selected for the certification process, using all available dPCR assays. Finally, dPCR can be used to measure the stability of the material over time to see if the concentration is decreasing (possibly due to degradation) or even increasing (possibly due to evaporation).