Counting DNA Molecule by Molecule

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Digital PCR is an established nucleic acid (NA) quantification technology, which enhances the broad use of PCR across molecular biology laboratories globally through improved precision and accuracy. Organizations that impact the quality of nucleic acid measurement and testing (metrology institutes, standards manufacturers, and quality assurance [QA]/proficiency testing organizations), adopted digital PCR early on and have since been pioneering its use for improved accuracy and precision in NA measurement. Ultimately, this will positively impact the quality of NA measurements generally and, more specifically, clinical NA testing. Molecular counting of nucleic acid molecules is the next measurement paradigm.

Digital PCR — A Technology Step Change
Today digital PCR is a well-established technology, which enhances the broad use of PCR across molecular biology laboratories globally. From early attempts at limiting dilution PCR (Saiki et al. 1988) to using 384-well plates (Vogelstein and Kinzler 1999), through to microfluidic chambers on the Fluidigm Systems to current emulsion (Bio-Rad Laboratories, Inc., RainDance Technologies, Inc., Stilla Technologies) and chip-/plate-based approaches (Thermo Fisher Scientific Inc., JN Medsys, QIAGEN), digital PCR has become a variant of PCR adopted by those looking for greater precision and accuracy than that afforded by other amplification techniques, such as quantitative PCR (qPCR) and its variants. Among this range of platforms and formats, Bio-Rad has become the leader in the field with thousands of QX200 and QX ONE Droplet Digital PCR (ddPCR) Systems worldwide and more than 4,700 peer-reviewed publications covering applications spanning disease testing and monitoring, gene editing, cell therapies, and feed and food testing across the whole organismal spectrum from humans to viruses.

Often researchers progress from qPCR and similar amplification techniques to Droplet Digital PCR because of its technical benefits. For example, the use of Droplet Digital PCR in liquid biopsy analysis of circulating tumor DNA is realistically only possible at the current levels of sensitivity due to the partitioning of thousands of individual DNA molecules into thousands of individual droplets. Rare variant–containing molecules can then be detected in isolation within individual droplets. Rare variant detection that was previously restricted to >5% with qPCR and next-generation sequencing (NGS) is now readily achievable at <0.1% in a single well with highly sensitive Droplet Digital PCR.

Sensitivities of <0.02% (Lamy et al. 2015, Link-Lenczowska et al. 2018) for rare variants are possible within the population of 20,000 droplet partitions on the QX200 ddPCR System when utilizing single-well analyses. Levels of sensitivity can be further increased by merging multiple wells into a meta-well in an additive fashion (Hindson et al. 2011). Droplet Digital PCR thus has an expandable sensitivity range by adapting the number of wells utilized per sample, a definite benefit over qPCR or NGS.
Applications that utilize the precision of amplifying 20,000 droplet reactions in parallel have become core techniques for Droplet Digital PCR. Copy number variants that were typically restricted to determining haploid vs. diploid variants with qPCR can now precisely identify those ranging from 1 copy to >20 copies at the level of individual genome increments. Investigating copy number variants associated with cancer development and progression, for example, HER2 monitoring in breast cancer, can be challenging and open to subjective interpretation. With Droplet Digital PCR, clinical interpretation of HER2 amplification between <2 copies vs. >2 copies is readily achievable (Garcia-Murillas et al. 2013, Suryavanshi et al. 2019).

Similarly, ascertaining copy number variants beyond haploid vs. diploid variants in germ line scenarios is achievable, for example, distinguishing SMN1 and SMN2 gene copy variations in newborns diagnosed with spinal muscular atrophy (Vidal-Folch et al. 2018, Chien et al. 2017) and identifying gene copy variation as a contributing factor in schizophrenia development (Marshall et al. 2017).

In additional research, the effort to quantify copy number variations (CNVs) in polyploidy plant species (Juoanin et al. 2020) and determine transgene copy number in plants (Collier et al. 2017) benefits greatly from the precision achievable with Droplet Digital PCR.

Ultimately bringing together both the sensitivity and precision of Droplet Digital PCR, CNV detection in rare genomic fractions is possible with noninvasive prenatal testing and graft vs. host disease detection in patients with transplants.

Detecting small amounts of fetal DNA is technically challenging and yet achievable within the large background of maternal DNA (typically 4–5% fetal) using Droplet Digital PCR. But being able to detect small changes (2 vs. 3 chromosome copies for fetal aneuploidy) within the fetal subpopulation of maternal DNA requires combined levels of precision and sensitivity unachievable with other PCR techniques. This application does require >20,000 droplets. But with the meta-well merging capabilities of Droplet Digital PCR described above, detection of fetal aneuploidy is readily achievable (El Khattabi et al. 2016, Lee and Hwang 2015).

Similarly, several different methods have been described to detect donor-derived circulating tumor DNA (ddctDNA) in the blood of recipients with transplants using Droplet Digital PCR. Goh et al. (2017) utilized donor vs. recipient discriminatory insertion-deletion mutations, Zou et al. (2017) utilized human leukocyte antigen–specific probe sets to identify ddctDNA, and Oellerich et al. (2014) utilized a comprehensive panel of single nucleotide polymorphisms to identify ddctDNA in recipient plasma. These methods again utilize the ddPCR technical benefits of increased precision and sensitivity over alternative PCR-based approaches to allow for the monitoring of small amounts of circulating DNA.

More recently, determination of viral copy number (VCN) of chimeric antigen receptor T (CAR-T) cells and T-cell receptor (TCR) cell lines by Droplet Digital PCR has been published on extensively (Fehse et al. 2020, Kao et al. 2018, Lu et al. 2020). Precision monitoring of adeno-associated virus and lentiviral integration into cells for downstream gene therapy use in human clinical treatments is a huge regulatory and clinical issue, as measuring VCN accurately is critical in producing the optimal dose for patients. From both technical and clinical perspectives there is growing acceptance that Droplet Digital PCR offers a fully viable route to determining VCN for clinical dosing of CAR-T cells and TCR cells within recommended safe ranges due to its absolute quantification capabilities and precision.

The Science of Measurement

In parallel with the research groups using Droplet Digital PCR to further our understanding of biology across the globe and enhance our clinical treatments of the human condition, there is a research community that underpins every measurement that is made in every laboratory. Metrology is the science of measurement and forms the foundation of the vast majority of processes that occur throughout a normal day, for example, the speed and distance you travel to work, the volume of coffee you buy on the way to work, and the weight and electrical power of the elevator that takes you up to your office! The majority of the units of measurement are founded on Système International (SI) units governed by the global measurement organization, the Bureau International des Poids et Mesures. Implementation of these units of measurement is monitored, managed, and promoted by metrology laboratories within each country.

In molecular biology and clinical and research studies, metrology labs were early adopters and fully implemented digital PCR with a focus on improving precision and accuracy in measuring nucleic acids. The biological application goal across global metrology labs, for example, for liquid biopsy variants, viral detection, and genetically modified organism (GMO) detection, is almost secondary to the more global goal of defining good measurement practices and the development of good reference materials. Each National Institute of Metrology tends to have a different biological focus, or several, but a common metrological focus to improve measurement processes and practices in science.

The National Measurement Institute of Australia has focused on the applications of nucleic acid copy number count, copy number variation, and absolute nucleic acid quantification, key techniques for which Droplet Digital PCR is well-suited. From the very earliest of papers on the use of Droplet Digital PCR
The National Institute of Biology in Slovenia has published several articles about the detection and quantification of genetically modified organisms (Dobnik et al. 2015, Kosir et al. 2019) and viral detection (Dobnik et al. 2019, Pavsic et al. 2017). This work highlighted that Droplet Digital PCR, a relatively new technology, can be heavily multiplexed with no loss of precision or accuracy compared to qPCR. It also demonstrated (with other metrology institutes) that Droplet Digital PCR has high reproducibility and repeatability, without the use of a standard curve, and, as such, is potentially suitable as a metrological reference method. These two factors (multiplexing and lack of standards) are significant for future laboratories’ adoption of Droplet Digital PCR in place of established qPCR. This latter point is well illustrated by the relatively early adoption of Droplet Digital PCR by groups aiming to determine VCN in the gene editing field.

In support of the use of Droplet Digital PCR for reference material validation, the National Institute of Standards and Technology (NIST) in the U.S. has utilized digital PCR to carry out extensive research on absolute quantification of both double- and single-stranded DNA (Duewer et al. 2018, Kline and Duewer 2017, Kline and Duewer 2019). Interestingly, although much of the work around Droplet Digital PCR has been focused on defining the technology as being suitable for traceable nucleic acid quantification, NIST also has been working with digital PCR in various forms for the development of reference standards over the last 8 years. Early microchamber digital PCR systems were utilized to develop viral reference materials for cytomegalovirus (Haynes 2013). More recently, ddPCR technologies were adopted to develop further reference materials for the BK virus (Cleveland et al. 2018) as well as in oncology for HER2 gene amplification detection and EGFR and MET gene copy number analysis (He et al. 2016, He et al. 2019), and in the development of an international reference material for quantification of human genomic DNA (Romso et al. 2018).

Similarly to NIST in the U.S., the Joint Research Center of the European Commission has been involved in metrological determination of reference materials and nucleic acid practices across a range of applications, including validation of GMO control plasmid for qPCR testing (Grohmann et al. 2017), copy number determination (Corbisier et al. 2015, Deprez et al. 2016), and liquid biopsy ctDNA analysis (Whale et al. 2018). But very early on in their adoption of digital PCR, these researchers highlighted the potential of the technique for analytical assessment of nucleic acids.

In the last 8 years, early publications by metrology labs have focused on assessing digital PCR for accurate and precise measurement of nucleic acids by comparing it to qPCR. Quantitative PCR has the potential for high precision but has limitations around accuracy and a large potential for bias (uncertainty) when using poor-quality standards for a standard curve. Droplet Digital PCR has become established as a technique with the precision to match qPCR and absolute accuracy better than that of qPCR. It provides absolute quantification without the need for standards.

Metrology experiments are often structured around high levels of intralab and interlab replicates to illustrate that Droplet Digital PCR is robust for comparison within and between labs, again without the need for common or international standards. Test samples are typically set up gravimetrically with the aim of reducing experimental uncertainty, thus ensuring accuracy, which leads to a better understanding of the underlying repeatability and reproducibility of the ddPCR technique. Finally, comparison to orthogonal technologies for measuring nucleic acids has taken Droplet Digital PCR closer to being defined as an independent, traceable technique for nucleic acid quantification (Whale et al. 2018, Yoo et al. 2016). This idea was also proposed by Mehle et al. (2018) with Droplet Digital PCR compared to qPCR and Kline and Duewer (2017) in their comparison of chip-based digital PCR (cdPCR) to Droplet Digital PCR.
Molecular Counting
By applying Droplet Digital PCR to a range of real-world application challenges, the National Measurement Laboratories in multiple countries have illustrated that digital PCR has the following properties:

- **Repeatability** — intralab studies illustrate the same answer, without the need for calibrators or standard curves
- **Reproducibility** — interlab studies illustrate the same answer, without standards or calibrators, or even the same assay design
- **Reduced uncertainty (absolute quantification)** — by comparison to orthogonal methods (for example, inductively coupled plasma–mass spectrometry and cdPCR), the molecular count answer is as close to truth as possible with minimal (and acceptable) uncertainty

And as an outcome of these technological capabilities Droplet Digital PCR has been accepted as a primary reference measurement procedure for SI traceable quantification of nucleic acid primary reference materials through molecular enumeration (ISO 20395:2019, ISO 17511:2020). By comparison, qPCR is recognized as a traceable method, but relies on suitable traceable reference materials.

Droplet Digital PCR can be used to simply count your DNA molecules (taking into consideration specificity, uncertainty, and completeness of count)!  

Manufacturers of Reference and Quality Control Standards
Besides the metrology labs aiming to define good metrological practices in molecular biology and rapidly adopting Droplet Digital PCR for that purpose in nucleic acid quantification, there are organizations that are utilizing the benefits of Droplet Digital PCR at a more applied level.

In the United Kingdom, the National Institute for Biological Standards and Controls (NIBSC) plays a major role in production and distribution of international reference materials, including the majority of World Health Organization (WHO) international standards and reference materials. In the context of nucleic acid detection and testing, NIBSC produces a wide range of viral and bacterial WHO controls that are defined based on the International Unit, an accepted arbitrary unit of definition. These controls are typically defined by a large interlab study using an extensive range of nucleic acid detection techniques to illustrate broad commutability of the control (NIBSC code 09/162: Fryer et al. 2016). NIBSC also produces genomic controls utilizing Droplet Digital PCR as a key technology during validation in recognition of its inherent precision and absolute quantitative capability for nucleic acids (NIBSC code 18/130: WHO International Standard, Sanzone et al. 2019).

Manufacturers of quality control (QC) standards have been relatively early adopters of Droplet Digital PCR, particularly in the areas of translational research and oncology. One of the major technical benefits of Droplet Digital PCR is the ability to precisely measure the proportion of a rare DNA sequence in the presence of a common variant. This is perfectly exemplified when monitoring somatic variants in the blood of patients with cancer. cfDNA can be present in low-integer numbers in a background of thousands of wild-type DNA molecules. Droplet Digital PCR is well adapted for detecting these rare fragments.

However, to validate this process in translational and clinical laboratories, controls must be utilized under current testing paradigms to validate the sensitivity of the ddPCR platform. To that end, companies such as SeraCare Life Sciences, SensID GmbH, and Horizon Discovery Ltd. have developed a range of oncology controls with mutant fractions applicable across a broad range (<0.1–10%) for this purpose and use Droplet Digital PCR as their QC technology. Exact Diagnostics, which produces control materials for the virology and bacteriology markets, utilizes Droplet Digital PCR for its precision and accuracy in the infectious disease field, where absolute accuracy and sensitivity are critical requirements.

External Quality Assurance and Proficiency Testing
Moving closer toward the clinical testing world, there is a recognition among external quality assurance (EQA) and proficiency testing (PT) organizations that Droplet Digital PCR may offer benefits for nucleic acid detection and quantification. This may come from the indirect benefit of buying EQA controls from an established QC manufacturer that has adopted Droplet Digital PCR (for example, SeraCare EGFR Panel for Genomics Quality Assessment [GenQA]) and, thus, providing higher-quality reference materials for an EQA. Or it may come from the direct adoption of Droplet Digital PCR into the development path of an EQA either during the production of internal EQA controls (for example, Quality Control for Molecular Diagnostics [QCMD]) or in the quantification of participant samples as part of an NA extraction EQA (GenQA). All of these approaches improve accuracy and precision across the EQA process.
The purpose of EQA organizations is not to promote individual technologies, but to assess the use of a range of technologies used by clinical labs and support those labs in achieving suitable QA accreditation. But recognition of the benefits of digital PCR over other nucleic acid amplification test technologies may present itself through the cumulative results generated over future EQA schemes. Further recognition of this benefit by participating clinical labs will ultimately result in better and safer clinical testing.

**The Next Steps for Absolute Nucleic Acid Quantification**

Absolute quantification by qPCR requires standards to generate a standard curve, of known quantity, which, ideally, is SI traceable. Variant allele frequencies determined by NGS require controls and calibrators that confirm mutant allele ratios and limits of detection and quantification. Since there is a large establishment of testing labs equipped with qPCR systems and NGS systems, accurate standards generated by Droplet Digital PCR do reduce overall measurement uncertainty in an SI traceable manner. While pragmatically accepting that many labs cannot discontinue old technology overnight for the next-generation technology, this approach does endorse Droplet Digital PCR as the core NA quantifying technology that subsequently supports the qPCR and NGS instrument/testing base.

Over time, many core labs may choose to adopt Droplet Digital PCR directly rather than obtain SI traceable standards for use as controls for second-generation qPCR technologies. There is already growing literature illustrating NGS labs utilizing Droplet Digital PCR as not only an orthogonal validation method but also indirectly as a measurement technique that is SI traceable.

**Conclusions**

Although a relatively unknown sector within molecular biology and clinical testing, it is vitally important that metrologists (who are molecular biologists) both validate new technologies for NA quantification and promote the benefits of those technologies if proven. It is relatively easy to understand that quantifying DNA (or RNA) using Droplet Digital PCR may improve downstream manipulation of samples in subsequent molecular biology experiments, for example, input sample amounts for NGS library preparation. However, the more important purpose lies in understanding how being able to quantify NA more accurately, as defined by National Measurement Laboratories, has a direct impact on standards manufacturers and EQA and PT organizations, and, ultimately, on clinical labs that are diagnosing human disease.

As Droplet Digital PCR becomes established as an independent reference measurement technology, its value as an orthogonal (SI traceable) validation technology for NGS also becomes recognized. This, in turn, impacts the quality of clinical testing by NGS panels. Beyond high value clinical testing (NGS validation and quantification of nucleic acids), where the benefits of adoption support the added costs of the technology over qPCR, adoption by clinical and research labs on a broader scale must balance the costs of adopting new technology against the benefits of improved precision and absolute quantification. Ultimately, there are opportunities for digital PCR to become a routine NA quantification method, in a format that is smaller, less expensive, and with a faster turnaround time than today.

There is recognition within the scientific community as a whole that Droplet Digital PCR offers improved accuracy and precision over qPCR, and this leads to the ability to determine low-level variant allele frequencies and precise CNV detection. But despite broad recognition of Droplet Digital PCR for independent absolute quantification, adoption is still not widespread, even though the majority of publications illustrating Droplet Digital PCR cite “absolute quantification without standards” as a major benefit over other NA amplification technologies in their introductions. This reticence to count molecules absolutely is illustrated clearly by the majority of translational research publications in oncology investigating somatic mutations and declaring the mutant copy number as a percentage or ratio to wild-type genomes rather than mutant copies per unit of blood or plasma. This may change if Droplet Digital PCR is accepted as a primary reference measurement procedure for SI traceable quantification of primary reference materials through molecular enumeration. This would open the door for Droplet Digital PCR to become a primary reference measurement procedure in the routine laboratory by molecular counting.


Vialle AS et al. (2016). International interlaboratory digital PCR study demonstrating high reproducibility for the measurement of a rare sequence variant. Anal Chem 89, 1,724–1,733.


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