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Digital PCR: Improving Nucleic Acid Quantification

Precision, Accuracy, and Sensitivity Are Among the Benefits Reported by Researchers

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Researchers at Harvard Medical School are studying the differences in human genomes to identify the genes underlying biological processes and human disease. One of the researchers, professor Steve McCarroll, is analyzing the copy number variations (CNV) of genome segments from tens to hundreds of thousands of base pairs long. This requires the ability to measure the precise copy number of these segments in thousands of individuals.

Using technologies such as real-time PCR (qPCR) and comparative genomic hybridization arrays, the McCarroll lab could measure simple deletions and duplications—changes in copy number from two to one or zero, or from two to three or four. But what they lacked was the ability to precisely and reproducibly measure copy numbers greater than four.

Bio-Rad Laboratories' (www.bio-rad.com) QX100 Droplet Digital PCR (ddPCR) system provides an absolute measure of target DNA and RNA molecules. It can be used to discriminate small-fold differences in copy number, enabling researchers to measure 1, 2, 3, 4, 5, 6, or more copies.

Whereas qPCR quantifies nucleic acids by comparing the number of amplification cycles and amount of PCR end-product to those of a reference sample, ddPCR enables researchers to directly quantify nucleic acids. In other words, ddPCR does not require the use of a standard curve.

Although qPCR is a viable detection strategy when mutant and wild-

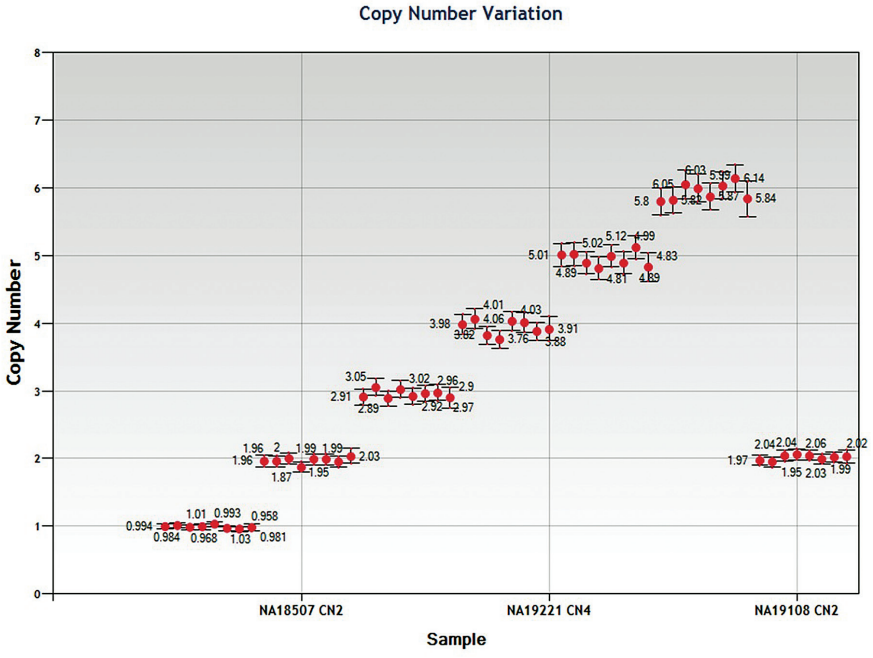

Figure 1. Copy number variation analysis using droplet digital PCR: Determination of MRGPRX1 copies per genome of seven different HapMap samples (Coriell). MRGPRX1 copy number values were normalized to the RPP30 reference gene using standard hydrolysis probe based assays. Error bars represent 95% confidence intervals.

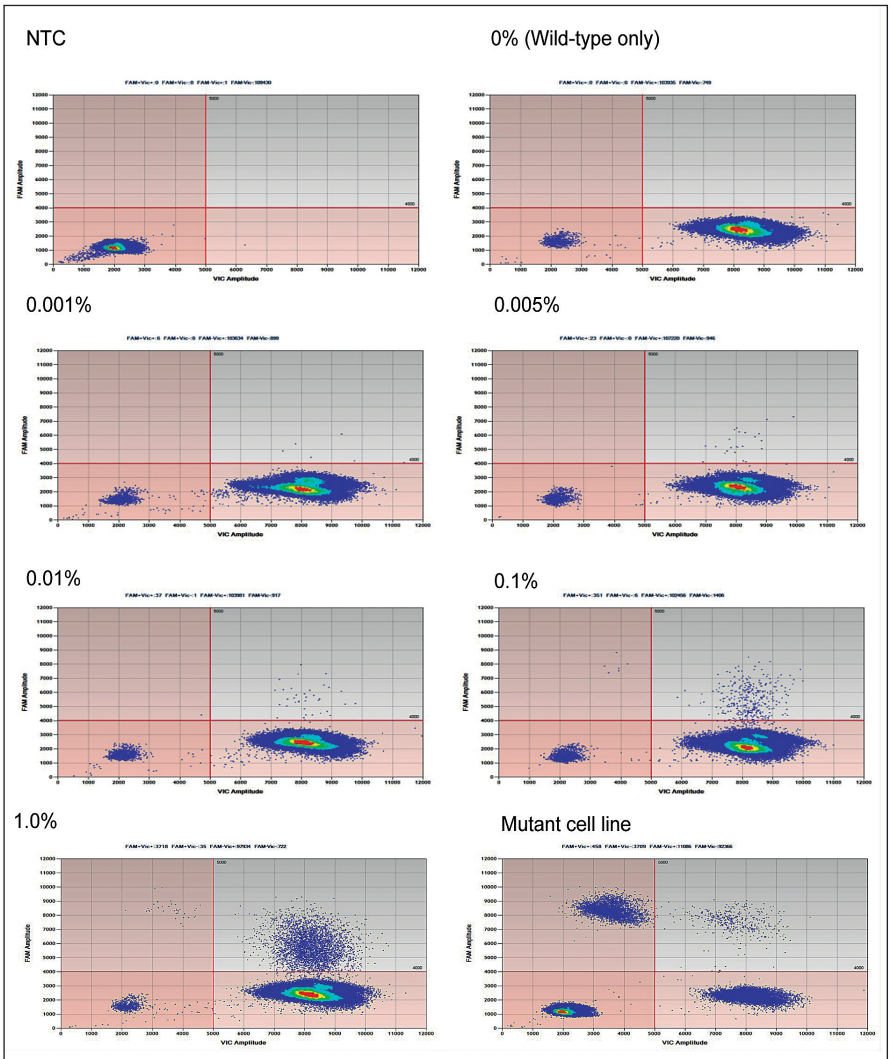

Figure 2. Droplet digital PCR detection of BRAF V600E rare mutant allele. Serial dilutions of mutant cell line ATCC HT29 in the presence of wild-type DNA using standard hydrolysis probe assays. High level of partitioning permits significant enrichment effects allowing detection of rare mutants at levels 1,000 times lower than with qPCR.

type sequences are mixed, its effectiveness declines when the mutated sequence is relatively rare, such as in myeloplastic syndromes (<20–25%).

Droplet Digital PCR separates samples into 20,000 droplets, and reactions are carried out individually in each. This reduces background interference for more reliable and sensitive measurement of low concentrations of nucleic acid that may not have been detectable using qPCR.

How ddPCR Works

Droplet Digital PCR takes advantage of simple microfluidic circuits and surfactant chemistries to divide a 20 µL mixture of sample and reagents into 20,000 droplets with target and background DNA randomly distributed among them. These droplets support PCR amplification of single template molecules using assay chemistries and workflows similar to those for qPCR applications (i.e., TaqMan).

After PCR amplification occurs, a reader determines which droplets contain a target and which do not. Software calculates the concentration of target DNA as copies per microliter from the fraction of positive reactions using Poisson statistics.

In digital PCR, having more partitions provides greater precision and resolution for detecting small concentration differences. Sample partitioning to levels above 10,000 allows for extremely accurate Poisson correlations and substantial enrichment effects when screening for rare events. The QX100 is the only digital PCR system that generates uniform droplets to partition a target sample. Others use microfluidic chips, which are challenging to scale to higher partition numbers per sample while maintaining low costs.

Gene-Expression Analysis

The arrival of qPCR revolutionized the field of gene expression, becoming the predominant technology for this type of research. With improved instrumentation and high-quality reagents, researchers are now able to report gene-expression levels varying at very fine amounts.

Unfortunately, resolution at levels below 50% are difficult to achieve due to the compounding of errors derived from each step in the quantification process. From a qPCR perspective, these include the dependence on a standard curve for amplification efficiency determination, standard error of this curve, technical replicate variation, normalization to reference genes (each of which carries previous errors), and comparisons between samples (e.g., normal and treated) that carry all these errors. Low expression targets tend to demonstrate greater variability between replicates due to the larger number of cycles required for amplification.

Droplet Digital PCR alleviates the compounding error effect, as readings are absolute; quantification is precise due to the digital nature of the assay and the fact that no standard curve is required. Normalization to multiple reference genes should still be performed (according to the MIQE guidelines) but here again, all values for these are standalone and not dependent on other samples or references, minimizing carried errors. Additionally, as a result of this inde-

pendence, when quantifying samples on different plates, different dates, or in different labs, the use of normalizing reference samples is no longer required.

Copy Number Variation Determination

CNVs include deletions, insertions, duplications, and complex amplifications. The accelerated discovery of CNVs has increased the need for high-throughput, low-cost options for validation and follow-up studies.

Traditional high-throughput technologies such as comparative genomic hybridization and single nucleotide polymorphism (SNP) arrays lack resolution and the ability to discriminate copy number differences of less than 50%. Quantitative PCR experiments require dozens of technical replicates for each target and reference gene in order to statistically discriminate between higher order variations.

The high resolution made possible by sample partitioning and digital analysis in ddPCR provides the precision necessary to resolve higher-order copy number states, making the QX100 ddPCR system the ideal solution for CNV validation while delivering the necessary throughput and cost efficiency.

Employing ddPCR, researchers have been able to completely resolve CNVs, distinguish less than 50% differences in gene copy, and accurately count genes that differ by only one nucleotide. For the HapMap MRG-PRX1 sample, copy number states from 1 up to 6 were completely resolved (*Figure 1*).

Rare Event Detection

Rare events include single nucleotide mutation, alteration of copy number, and deletion or insertion of nucleotides. These genetic variant molecules are difficult to detect due to their dilution by normal cells from either tissues or bodily fluids such as blood. Early detection of rare events can make all the difference in the outcome of cancer patients, as well as lead to more sensitive and less invasive diagnostics.

During qPCR, rare and normal variants of DNA molecules are amplified at an equivalent rate. If the normal gene is initially present in 100-fold abundance over the mutated gene, at the end of the reaction, 1% of the total amplification product will be the mutated gene amplicon. Its detection will be extremely difficult as the total signal generated will be 1% of that generated by the wild-type gene amplicon.

For rare event detection, sample partitioning increases sensitivity by distributing both mutant and normal genes into a large number of isolated reaction compartments. In each one of these isolated droplets, the rare mutant molecules are now at a more favorable ratio compared to the wild-type, and thus become detectable within that individual droplet. The QX100 can detect amplifications even in highly heterogeneous matrices where only a fraction of the cells are affected. This precision enables the detection of somatic copy number alteration—the hallmark of many cancers.

The detection of point mutations requires a high degree of sensitivity. Droplet Digital PCR allows the detection of 0.001% mutation fractions, as demonstrated in a duplex PCR reaction using TaqMan probes targeting the BRAF 600E mutation (*Figure 2*). This detection limit is more than 1,000 times lower than qPCR.

Looking Ahead with the McCarroll Lab

When McCarroll's lab acquired the QX100 ddPCR system, they immediately began using the instrument to study structurally complex regions of the genome—regions that have historically been influenced by many different structural mutations. In such regions, ddPCR has allowed them to quantitate copy numbers in large populations, which is important in both population genetic analysis and disease analysis. Now they can analyze these regions in the same high-quality, high-precision way that is the standard for genetic analysis of simpler kinds of variation such as SNPs. GEN

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