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Positioning Digital PCR for Sharper Genomic Views

To Get Even More Out of Weak Signals, dPCR Is Deploying Precision Fluidics, Selective Primers, and Powerful Analytics

Kate Marusina, Ph.D.

Recent developments in digital PCR technology allow for highly sensitive detection of rare events and discrimination of very minute genomic and transcriptomic changes. [jxfzsy/Getty Images]

Polymerase chain reaction (PCR), now one of the brightest lights in molecular biology's firmament, reached new heights of performance with skyrocketing speed. At first, when it struggled to achieve liftoff, PCR was a labor-intensive operation, requiring reaction tubes to be transferred from one temperature-controlled bath to another to accomplish denaturation, annealing, and elongation.

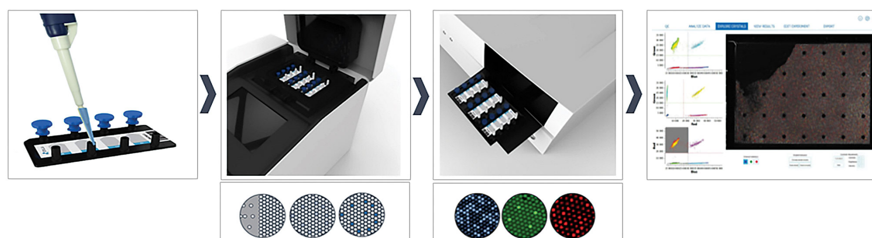
Soon, however, this approach to DNA amplification began an impressive upward climb, and PCR really began to accelerate after it was incorporated into the most sophisticated microchip technologies. By now, PCR has reached an orbit that is as stable as it is lofty.

PCR has sprouted multiple modules, each one becoming its own scientific domain. Quantitative PCR (qPCR) quickly became a widely adopted method for quantitating relative abundance of the targets. Digital PCR (dPCR) is the latest addition. It is particularly suited for absolute quantitation of target DNA, and specifically, for rare-event detection.

dPCR works by partitioning (diluting) a DNA sample into many individual, parallel PCR reactions, where some of these reactions contain the target molecule while others do not. Highly specific fluorescent probes bind to the amplicons differentiating between alleles. The number of droplets that light up with the same fluorescent signature provides an accurate measure of the number of target molecules in the original sample.

“Oncology drives the digital PCR field,” says Alexandra Martin, Ph.D., sales application specialist, Stilla Technologies (Paris, France). “dPCR is essential for detecting rare genetic variants present in very small quantities amongst a large number of wild-type copies. Specific, consistent detection of cancer alleles in blood samples would revolutionize diagnosis and personalized cancer treatment.

“Unfortunately, dPCR has been cumbersome to use,” Dr. Martin adds. “Our goal is to commodify dPCR, and make it as commonplace as any other PCR technique.”



At the heart of Stilla’s Naica System™ is the Sapphire Chip, a fully integrated microfluidic consumable. Both droplet generation and PCR amplification take place in the Naica Geode, an instrument that combines a flat-block thermocycler with a pressure chamber (*Figure 1*).

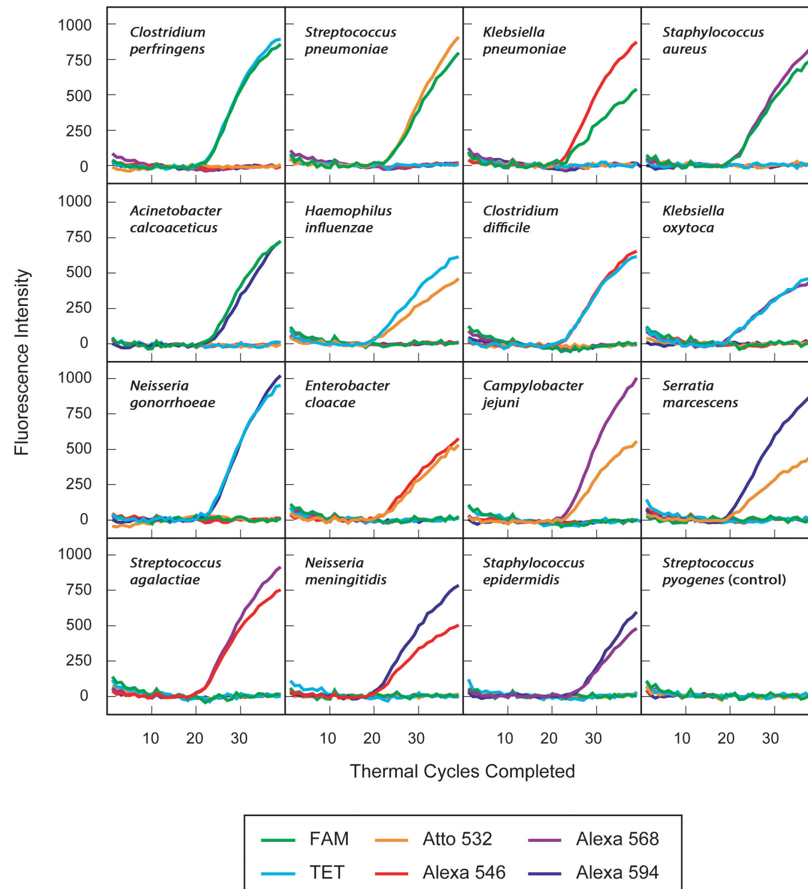
As the pressure steadily increases, the PCR mix flows through the droplet injection nozzles of the chip. The aqueous mix spontaneously breaks down into monodisperse droplets, which are propelled by surface tension into the oil-filled microfluidic chamber. Dr. Martin explains that droplets form a regular monolayer similar to that of atoms inside crystals, hence the name of the process: Crystal Digital™ PCR. Fluorescent imaging is done using the Naica Prism3, an automated fluorescence microscope equipped with three detection channels.

“Tight integration of all necessary technologies, minimization of pipetting, and automation decreased the time from initiation to result to only 2.5 hours,” asserts Dr. Martin. “Crystal Digital PCR is just as fast as any common PCR-based diagnostic method, and is able to efficiently differentiate single nucleotide polymorphisms.”

Naica Prism3 acquires a set of three high-resolution images for each droplet crystal through the transparent bottom surface of the Sapphire chips, with one image for each fluorescent channel (blue, green, and red). In a proof-of-concept study, a multiplex assay detected four common EGFR mutations in patients diagnosed with non-small cell lung cancer. A few follow-up samples presented an opportunity to visualize longitudinal correlation between treatment, mutations and clinical outcomes.

“Crystal dPCR detected mutations with higher sensitivity than NGS [next-generation sequencing]. It is also well suited to clinical use in terms of cost and time to results,” suggests Dr. Martin. In the next few years, Stilla plans to double the number of detectable colors to further expand multiplexing capabilities.

SuperSelective Primers Zoom in on the Somatic Mutations



"Somatic mutations characterize cancer cells," says Fred Russell Kramer, Ph.D., professor of microbiology, biochemistry, and molecular genetics at Rutgers University. "Liquid biopsy, or genetic analysis of a blood sample, is the only practical way to detect newly arising somatic mutations" (*Figure 2*).

Dr. Kramer's team creates DNA amplification primers of a highly innovative design. These primers, called SuperSelective primers, incorporate both a relatively long 5' "anchor" sequence that hybridizes strongly to target DNA fragments, and a very short 3' "foot" sequence. The foot sequence contains an interrogating nucleotide—the one that matches the target somatic mutation. Thus, this sequence is perfectly complementary to the mutant template, but mismatches the related wild-type sequence. A "bridge" sequence lies between the anchor and the foot.

When the SuperSelective primer is hybridized to a template molecule, the bridge sequence and the template form a "bubble" that functionally separates the strong anchor hybrid from the weaker foot hybrid. The extraordinary selectivity of SuperSelective primers comes from preferential and efficient amplification of perfectly matched foot-template hybrids.

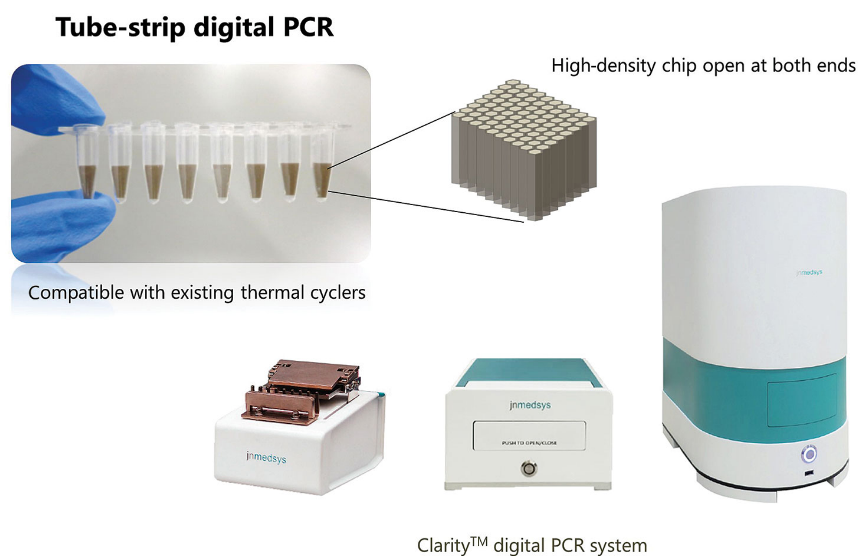
"We have learned how to optimize the design of SuperSelective primers, such as the length of the foot and the length and symmetry of the bridge, and we are now able to reliably detect 10 mutant DNA fragments in the presence of one million related wild-type DNA fragments, even though the only difference between the mutant and the wild-type is a single nucleotide polymorphism," emphasizes Dr.

Kramer. “This means that a sample analyzed by digital PCR does not have to be diluted as much. Instead of millions of droplets, we can go down to just 25,000, and yet, the wild-type fragments are not amplified.”

Another SuperSelective innovation is a unique 5' tag sequence corresponding to a somatic mutation to be detected. These tag sequences are identified with fluorescent molecular beacons, each labeled in two unique colors from a palette of six different colors detectable by Stilla Technologies’ Naica platform. The two colors in a dPCR droplet unequivocally indicate the presence of a given variant.

“With six fluorescent channels incorporated into the upcoming Stilla Naica reader, duplex color-coded molecular beacons can be used to identify 15 different somatic mutations, and triplex color-coded molecular beacons can detect 20 somatic mutations, from just 10 mL of blood,” adds Dr. Kramer.

“Microchip in the Tube” Combines the Best of Both dPCR Worlds



Circulating cell-free (cf) Epstein–Barr virus (EBV) DNA, which is an effective biomarker for nasopharyngeal carcinoma (NPC), has been shown to correlate with NPC prognosis and prediction of treatment outcomes. However, cfDNA is scarce and short-lived, so it can be hard to incorporate into diagnostic and prognostic procedures.

Clarity™ Digital PCR System (JN Medsys, Singapore) was used to detect and quantify EBV cfDNA from patient plasma (*Figure 3*). “EBNA-I is a single-copy gene in the EBV genome, and thus, is ideal for viral-load quantification,” notes Erin Zhang, Ph.D., product manager, JN Medsys. “But its levels as circulating cell-free DNA (cfDNA) are very low.”

In a recent study,[1] Clarity produced high detection rate of EBNA-I, highlighting the superior sensitivity among the platforms tested. The data correlated well with clinical outcomes, as several EBV-positive cases uniquely detected by Clarity dPCR relapsed soon after. Dr. Zhang mentioned typical challenges

associated with droplet dPCR, such as coalescence of droplets and significant sample loss. On the other hand, chip-based dPCR needs a dedicated thermocycler.

“The Clarity system combines the best of both droplet- and chip-based dPCR technologies to bring high-throughput and easy-to-use system that can be leveraged for a variety of applications,” adds Dr. Zhang. The Clarity system utilizes a strip of eight standard PCR tubes. A miniature trapezoidal-shaped chip consisting of 10,000 partitions is fabricated inside each tube. As a mix of qPCR reagents, primers, probes, and the sample is introduced into each tube, the liquid is rapidly drawn into the partitions by capillary action.

The partitions are then sealed with a sealing fluid, and PCR reaction is performed using a conventional thermocycler. The closed, sealed environment prevents cross-contamination between samples.

“Clarity is an open-platform system,” adds Dr. Zhang. “It is compatible with existing qPCR reagents. The workflow is also similar to a conventional PCR. Users can get started without significant training. The tube-strips are easy to handle and are compatible with most conventional thermocyclers.”

In a unique application,[2] Clarity was used to quantify pathogenic bacteria in treated wastewater after purification with anaerobic and aerobic membrane bioreactor methods. “In another example,[3] the Clarity System was utilized to assess the decrease in viral load in treated wastewater,” says Dr. Zhang. JN Medsys plans to expand its marketing efforts to the United States.

Coevolution of Reagents and Instrumentation for Optimal Performance

“Digital PCR has become a mainstream technology and has overcome many technical hurdles from its inception,” says Jennifer Jackson, Ph.D., market development scientist, digital biology group, Bio-Rad Laboratories (Billerica, MA). She explains that fine-tuning assay design for droplet digital PCR (ddPCR) required a paradigm shift. The unparalleled sensitivity and specificity of ddPCR revealed imperfections in sample quality and PCR assays that are otherwise not seen in bulk qPCR reactions.

“Early testing of existing qPCR assays on the ddPCR system exposed performance issues, including probe cross-reactivity resulting in background noise. Based on these early observations, Bio-Rad committed to developing highly specific assays for use on the ddPCR platform,” adds Dr. Jackson.

As a result of these efforts, 200 validated point-mutation detection, 700 validated copy-number variation, and more than 60,000 gene-expression assays are now commercially available. Additionally, one of the innovations to support customers’ assay needs was the development of a design portal based on unique algorithms for perfecting dPCR primers and probes. This online tool allows scientists to employ the same algorithms used to design Bio-Rad’s own commercial assays.

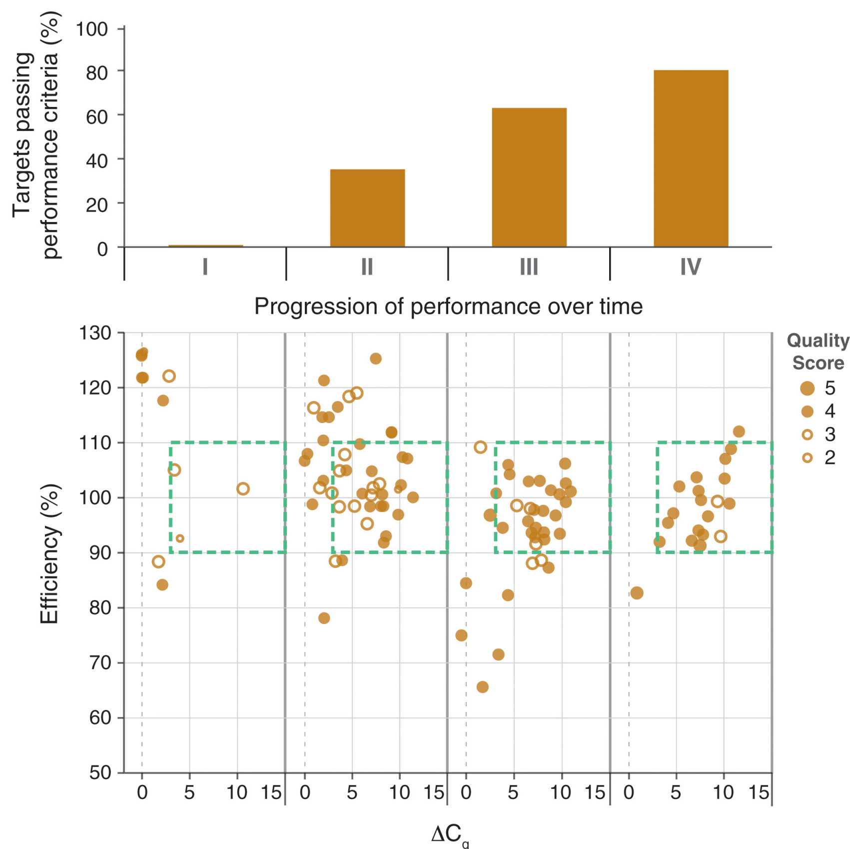
“To provide a comprehensive ddPCR solution, we coevolved our reagents with the instrumentation,” continues Dr. Jackson. “QX200 is our most advanced second-generation platform consisting of a droplet generator (or an automated droplet generator, AutoDG™) and a droplet reader. Altogether, these

developments allow for highly sensitive detection of rare events and discrimination of very minute genomic and transcriptomic changes."

As an example, assessing KRAS mutations has been challenging due to multiple single nucleotide polymorphisms (SNPs) and low variant allele frequency. To optimize detection strategies, Bio-Rad developed the ddPCR™ KRAS Screening Multiplex Kit. In one study, a panel of seven actionable KRAS mutations from FFPE samples was screened using the QX200 platform. The assay detected KRAS mutations present at <1% fractional abundance and showed excellent concordance between multiplex and duplex detection.

Dr. Jackson also highlights that the QuantaSoft™ Analysis Pro Software significantly simplifies multiplex assay analysis, and is unique in its ability to deconvolute fluorescent signals from more than two targets of interest. Bio-Rad continues extending this technology into clinical diagnostics obtaining the CE-IVD markings for the QX200 and AutoDG systems in Europe and Asia.

"Dots in Boxes" for Meta-Comparison of qPCR Reactions



"New England Biolabs [NEB] takes pride in creating reliable and practical molecular biology tools," says Nicole Nichols, Ph.D., group leader, amplification development, NEB. "Entering the qPCR space as late as we were, there was no room for being mediocre."

With qPCR firmly established as an essential technique for detecting and quantifying nucleic acids, the reagent market was overflowing with a multitude of options, creating a confusing array of choices.

"Comparing all existing product offerings quickly became a complicated task," notes Dr. Nichols. "We began by developing a visualization tool for meaningful comparisons between reagents available on the market."

In the past decades, a diverse set of protocols, instruments, and analysis methods generated volumes of qPCR data—data not easily amenable to meta-comparisons. The need for consensus on best practices led to establishment of optimal experimental guidelines, or Minimum Information for Publication of Quantitative Real PCR Experiments (MIQE). The guidelines defined several performance metrics to ensure assay robustness and reproducibility, such as PCR efficiency, dynamic range, limit of detection, target specificity, and precision.

MIQE guidelines are essential when developing a new product for conducting and reporting qPCR experiments. However, quantity of experimental data quickly becomes overwhelming, and qPCR curves are difficult to analyze for subtle changes in product performance. Dr. Nichols explains that in addition to the basic MIQE guidelines, NEB wanted to analyze an additional set of quality characteristics, such as curve reproducibility, shape, and steepness.

NEB adapted their laboratory information management system (LIMS) to collect detailed input and output data. "Now we had an ability to correlate changes in inputs with qPCR outcomes, and we used MIQE along with other quality parameters to analyze the dataset," adds Dr. Nichols.

Visualization of large datasets presented significant challenges, according to Dr. Nichols. A high-throughput data analysis method termed "Dots in Boxes" summarizes and captures results of 18 qPCR reactions (triplicate dilutions plus controls) as a single dot in a two-dimensional plot (*Figure 4*). The size and opacity of the dot correlates with the quality score. Systematic variations in composition of qPCR reactions result in subtle changes in curves, which in turn affects positions and sizes of the dots, shifting the values in and out of the boxes defined by the typically accepted values.

The "Dots in Boxes" analysis and method visualization tool supported development of robust Luna qPCR kits. Scientists from the University of Georgia have used an NEB Luna One-Step RT-qPCR kit to detect low levels of bacterial mRNA among larger quantities of plant RNAs, where inhibition and sensitivity have previously been problematic for their experiments. The optimized kits support research in plant and microorganism communication.

Data were collected for qPCR targets varying in length and gas chromatography (GC) content, using Jurkat genomic DNA as input. Results were evaluated for efficiency, low input detection, and lack of non-template amplification (where ΔC_q = average C_q of non-template control–average C_q of lowest input). In addition, consistency, reproducibility, and overall curve quality were assessed. Dots in boxes permitted large volumes of data to be compared over multiple master mix compositions, ultimately driving reagent optimization (*Figure 4*). Progression of performance is displayed in *Figure 4* for several predecessors of the Luna Universal qPCR Master Mix (NEB #M3003). Mixes with successful qPCR performance were built upon to establish the final composition of the Luna products.

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