Digital PCR and real-time PCR: a time and place for each

When it comes to DNA research, scientists often find themselves weighing digital PCR (dPCR) against the more established quantitative real-time PCR (qPCR). Which technique is better to use? The answer is both. That's according to one Belgium-based PCR data analysis company that faces this question with increasing frequency these days.

By Dr George Karlin-Neumann and Dr Rachel Scott

Biogazelle NV, a Belgian-based spin-out company from Ghent University, has used qPCR as a core technology for more than a decade and still considers it to be the gold standard for gene expression analysis due its high-throughput and low costs. But the company also recently began using dPCR after purchasing a system in response to rising demand from researchers interested in cancer mutations, copy number variation analysis and rare event detection.

Both tools are assets in the lab, Biogazelle says. On one hand, they value qPCR for its known workflows and the Minimum Information for the Publication of Quantitative Real-time PCR Experiments (MIQE[1]) guidelines which help ensure that scientists get high quality, reproducible results from their qPCR experiments.

Digital PCR, on the other hand, delivers an absolute measure of target nucleic acid molecules, rather than the relative measure obtained from qPCR. The absolute DNA quantitation allows for precision, reproducibility and sensitivity, which enables researchers to measure smaller differences and accurately quantify minor variants in the background of a wild type sequence.

Recently, Biogazelle used dPCR to help a biotech firm design a PCR-based experiment for transgene copy number variation. Earlier attempts using qPCR did not allow detection of fold-changes lower than 50%, resulting in poor-quality data that was unusable. Yet dPCR was able to detect the smaller differences in copy number, providing more precise and reliable data.

Biogazelle now sees a place in its labs for both qPCR and dPCR. Its researchers decide on which system to use based on what type of results their clients are seeking.

But Biogazelle isn't the only company finding its balance in the expanding world of PCR. Here, we take a look at the state of PCR and share how other companies are addressing the question of which PCR technology is right for them.

Digital drives the PCR market upward

The combined qPCR and dPCR instrumentation market is expected to grow at a compound annual rate of 7.1% from 2011 to 2016, thanks in large part to the ramp up of the dPCR market, according to a recent industry report[2] from research firm Frost & Sullivan. In fact, last year researchers published nearly 100 papers with findings based on dPCR technology, according to PubMed (**Figure 1**).

As new competitors continue to enter the dPCR market, the average list price of $136,500 for a system (as of November 2012 when the Frost & Sullivan report was written) is likely to fall considerably over the next five years. Right now, cancer mutation detection, structural variation such as

copy number variation and rearrangements, and absolute DNA quantification are the three major applications facilitated by the technology.

However, suppliers are hoping to make their instruments applicable to a wider customer base, including the molecular diagnostics and applied markets. Digital PCR is highly suited for early detection of mutations associated with cancer and other diseases, which may alter the approach to treatment. This technology may also be used for biomarker discovery, non-invasive prenatal screening, tumour profiling and monitoring of residual disease. Frost & Sullivan believes applications in clinical and translational research will likely take a faster route to success due to its strong implications for better patient outcomes.

A lasting role seen for qPCR

Even as dPCR expands, suppliers trust that qPCR will remain a workhorse technology in molecular biology research.

Why? First, qPCR has been well-established as a credible and capable technology and is relied upon by researchers for its speed, sensitivity, specificity and ease-of-use. It is most useful for relative gene expression experiments and as a validation tool supporting other genomic methods including DNA microarrays and next generation sequencing (NGS) applications.

Also, because qPCR has been around since 1993, most researchers have easy access to the technology and to a large body of literature available for reference. Researchers can draw from their own historical data when designing and interpreting their experiments in gene expression analysis, genotyping, pathogen detection, viral quantification, DNA methylation analysis and high resolution melting analysis, among others.

As its name suggests, real-time PCR measures PCR amplification as it occurs. The relative nature of qPCR, which calculates a target's concentration or relative abundance by comparing it to a known concentration or control, makes the method particularly well-suited to gene expression analysis. When using qPCR, as is common with many other biological tools, changes in target expression results are most meaningful when compared with different experimental conditions, such as diseased versus healthy tissue.

Well-designed qPCR assays can detect several to as many as millions of copies of a target sequence per reaction giving it a considerable dynamic range. It is appropriate for screening or downstream validation experiments with its ability to detect targets with very low and very high copy number in the same run.

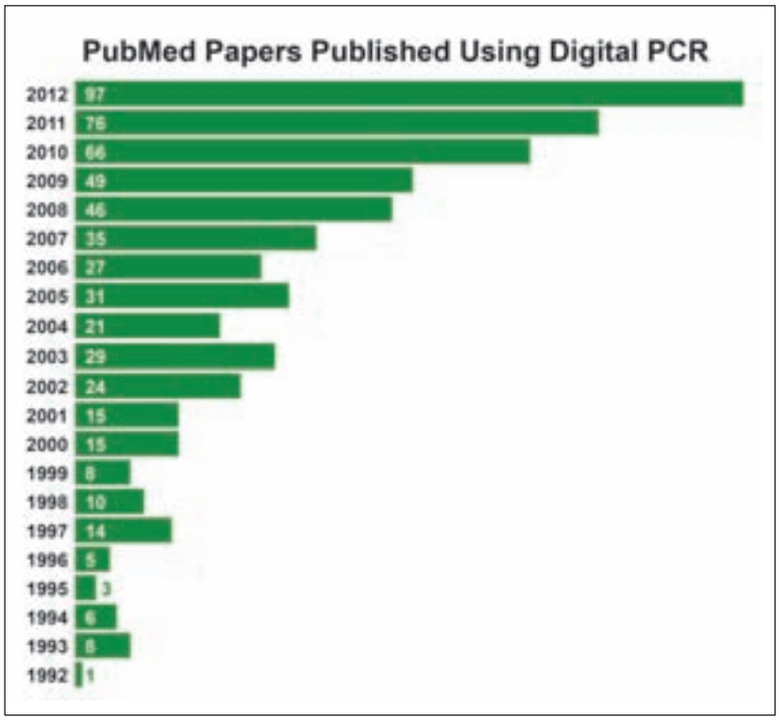

Figure 1
Growth in digital PCR papers from the year digital PCR was first developed

When using qPCR, researchers can choose their detection chemistry. They can select from inexpensive intercalating dyes (such as SYBR green) to a variety of target-specific probes (TaqMan, molecular beacons, etc). Per sample pricing is highly flexible since users can easily change reaction volume, throughput and detection method to meet experimental needs.

Researchers value precision of dPCR

Digital PCR is a nucleic acid molecule counting method that works by partitioning the sample to such an extent that only a small number of DNA molecules of interest (targets) are present in each partition, with numerous reactions run in parallel. This partitioning happens either by dividing the sample into chambers (cdPCR) or droplets (ddPCR). Determining the starting number of template molecules in the sample is possible by counting the number of positive and negative reactions at the end of the PCR.

Digital PCR provides an absolute measurement and, unlike qPCR, does not require the user to compare an unknown to a standard, thus eliminating the need for a standard curve. Eliminating the standard curve reduces error and improves precision. This enhanced precision enables day-to-day and lab-to-lab reproducibility, a feature that's only now beginning to be appreciated. In addition,

because samples are partitioned, the background DNA is reduced, lessening competitive effects and allowing for greater discrimination between similar sequences.

Two important dPCR applications are the measurement of gene copy number (referred to as copy number variation or CNV) and rare event detection, also known as RED. Rare event detection is useful when studying cancer gene mutations (diagnosis, monitoring therapy response, early relapse detection), infectious diseases (pathogen detection), GMO detection in food, and prenatal testing of genetic diseases. In reality, any study that requires higher resolution or higher relative sensitivity could benefit from dPCR.

Arriving in late 2011 as the latest commercial dPCR system, droplet digital PCR™ (ddPCR™) has been rapidly adopted by life science researchers due to its flexibility, enhanced performance, higher throughput and lower costs. Droplet digital PCR gets its name because it partitions samples into droplets prior to PCR amplification.

In 2012, Frost & Sullivan reported that 56% of healthcare-related laboratory decision makers in North America said they plan to install ddPCR systems in their labs within the next 12 months.

Applications abound: droplet digital PCR propels research in many fields

To understand what's driving demand of new PCR technologies, consider the diverse array of applications where ddPCR is fuelling scientific discovery today.

Detecting rare events

Most conventional methods of detecting somatic mutations have poor selectivity and fail to detect mutant sequences when they are present at less than 1%. However, during a presentation at the American Association for Cancer Research Annual Meeting in 2012[3], researchers demonstrated the detection of a mutation in the BRAF gene with sensitivity greater than 0.01%, or detection of one mutant target in the presence of 10,000 wildtype DNA molecules. The enhanced sensitivity offered by ddPCR technology enables the detection of somatic mutations with high selectivity and sensitivity, promising earlier and less invasive diagnosis of disease.

Guiding new clinical tests

Digital PCR has come to the rescue of many stalled qPCR studies. One such instance recently occurred at Monoquant, a molecular diagnostics firm founded by researchers at Flinders University and Medical Center in South Australia. Their goal was to isolate and quantify the abundance of a mutation common in chronic myeloid leukemia (CML) DNA to develop a new clinical test. Droplet digital PCR technology allowed the team to overcome variations in qPCR amplification efficiency, which affected quantitation accuracy. The company believes that the results they are seeing from their ddPCR system will fast-track FDA approval process for the test. This could offer patients a better degree of monitoring and better disease management by tracking the progression or remission of CML.

HIV eradication

The need to find a cure for HIV has spurred demand for new assays that can monitor the disease in infected patients who are taking combination antiretroviral therapy. These assays must offer high sensitivity, specificity and reproducibility, all while being cost-effective. Current assays offer high sensitivity or specificity, but not both. A new study[4] led by Matthew Strain and Douglas Richman of University of California, San Diego, found that ddPCR may be a suitable alternative to the most frequently used technique, qPCR. When using identical quantities of clinical samples from peripheral blood, ddPCR's precision was four-fold to more than 20-fold better than qPCR. Features such as improved sensitivity and precision make ddPCR particularly well-suited for measuring of the size of the latent HIV reservoir. Findings suggest it could be useful for clinical studies aimed at eradication of HIV from infected patients.

Understanding immortality

Telomeres, the protective structures at the ends of chromosomes, naturally degrade with each cell division. Once they fall below a critical length, the cells arrest and become senescent before dying or passing through a crisis phase and becoming immortalised cancer cells. One of the mechanisms of immortality is the activation of the telomerase enzyme, which adds a specific sequence of nucleotide repeats to telomeres. This rewinds the clock and enables a cell to divide continuously. Researchers are investigating telomerase activity as a biomarker for cancer diagnosis and as a target for anticancer drugs.

The presence of active telomerase is traditionally measured by the telomerase repeat amplification protocol (TRAP) assay by measuring the enzyme's activity on a starting DNA template, which is then amplified by PCR. However, researchers from the University of California, San Francisco, and Bio-Rad Laboratories found that ddPCR is significantly more sensitive than traditional TRAP assay and is more

amenable to high-throughput analysis. The technology's sensitivity enables the measurement of telomerase activity in samples below the detection limit of traditional methods, while also providing a quick and facile method of measurement in cells where telomerase is more highly expressed. This new ddPCR TRAP telomerase activity assay may enhance our understanding of telomerases' role in oncogenesis and other cellular and physiological phenotypes.

Analysis of GMOs

Recently, ddPCR has shown to be a feasible alternative to qPCR in the analysis of genetically modified organisms (GMOs). More than 60 countries representing 40% of the world's population require labelling of food and feed when GMOs reach certain thresholds. Screening for and quantifying GMOs are essential to the integrity of this labelling policy, and qPCR is the gold standard technique. However, Dany Morisset, a researcher at Slovenia's National Institute of Biology, says qPCR is often unreliable for quantifying very small numbers of DNA targets and is not ideal when targets are part of complex matrices such as foods or feed that contain inhibitory substances. In a recent study published in PLOS ONE[5], Morisset demonstrated that ddPCR could replace or be a good alternative to qPCR for quantifying the presence of GMOs. His study found ddPCR to be more accurate and reliable, especially when GMOs were at low levels. ddPCR methods also meet international food standards of applicability, such as being able to work across a wide range of food, and practicality, being that ddPCR is simpler to set up, involves less hands-on labour and is less expensive than qPCR, especially at higher sample throughput.

Furthering diagnostics

The research group headed by Hanlee Ji, an assistant professor at Stanford University School of Medicine, focuses on translational and clinical questions of cancer genetics that, once answered, have the potential to improve cancer patient care. Using ddPCR, Ji and team have developed numerous methods for the accurate interrogation of cancer genomes that overcome challenges associated with clinical samples and the genetic variability resulting from tumour evolution. ddPCR's ease of use, superior performance and rapid development time for novel assays give it potential for future diagnostic application.

Where is PCR headed?

PCR technologies as a whole – real-time and digital – show significant promise for growth in the future. While qPCR continues to serve a very real purpose in research labs, dPCR's impressive levels of sensitivity, precision, robustness and reproducibility are helping researchers propel their work beyond previous limits. Companies and researchers alike are adapting to the changing environment and finding their perfect balance in the world of PCR. DDW

Comparative Strengths of qPCR and dPCR

Strengths of qPCR	Strengths of dPCR
Established technology	Emerging technology
Relative measurement, ideally suited for gene expression analysis	Absolute measurement eliminates need for standard curve
Wide choice in detection chemistry and reaction volume equates to flexible running costs	High precision for better reproducibility for low input target concentrations
Large dynamic range	Greater sensitivity for rare mutation detection
Higher throughput, automation compatibility	Improved precision for higher copy number variation analysis

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Dr Rachel Scott *is the Group Marketing Manager for PCR instruments at Bio-Rad laboratories where she markets innovative PCR and qPCR instruments and software. Prior to her current position, she supported customers through applications and technical sales support. She received her PhD in molecular biology from Monash University, Melbourne, Australia.*

References

1 Bustin, SA et al. The MIQE Guidelines: minimum information for publication of quantitative real-time PCR experiments. PubMed, April 2009.

2 Frost & Sullivan. Market penetration leadership award quantitative and digital PCR instrumentation North American, 2012. Frost & Sullivan Best Practices Research, November 2012.

3 Hindson, B, Karlin-Neumann, G et al. Ultra-sensitive detection of rare mutants by droplet digital PCR with conventional TaqMan assays. April 15, 2012. AACR, Chicago, IL.

4 Strain, MC, Richman, DD. New assays for monitoring residual HIV burden in effectively treated individuals. Lippincott Williams & Wilkins 2013.

5 Morisset, D et al. Quantitative Analysis of Food and Feed Samples with Droplet Digital PCR. PLoS ONE, May 2013.