

PCR ASSAYS CAPTURE GENE EXPRESSION INSIGHTS



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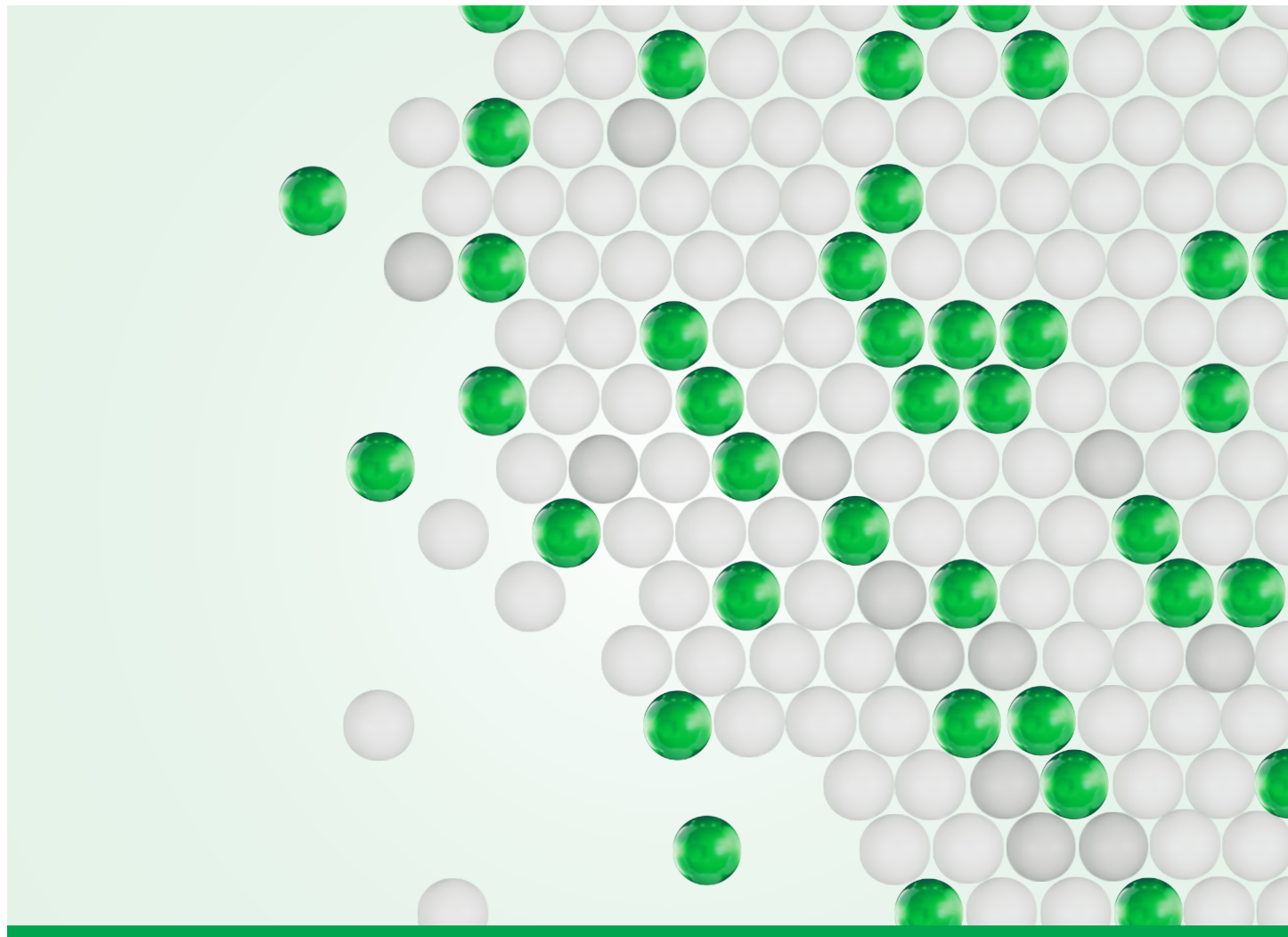
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GENE EXPRESSION ASSAYS THROUGH THE AGES



Assays that enable researchers to measure gene expression have long been central to understanding both normal physiology and disease. In oncology, for instance, dysregulated oncogene or tumor suppressor expression can drive malignant transformation, tumor growth, and metastasis.¹ In neuroscience, changes in the expression of neurotransmitter receptors, ion channels, or growth factors influence processes such as synaptic plasticity, memory, and neurodegeneration.² Reliable and scalable methods to quantify expression have become increasingly crucial across laboratories, and over the past three decades, polymerase chain reaction (PCR)-based techniques have transformed how scientists analyze transcripts, as well as their downstream translational applications.³

The Background of PCR-Based Methods

Developed in the early 1980s, conventional PCR or end-point PCR is often regarded as one of the earliest genetic technologies that researchers adopted for gene expression analysis.³ This approach allows scientists to amplify DNA or RNA-derived complementary DNA (cDNA) to visually detect expression, usually via gel electrophoresis. Conventional PCR provides greater sensitivity compared to microscopy-based hybridization methods, but it remains largely qualitative. Researchers can now use digital tools to compare signal intensities, but these results are typically semi-quantitative.

The introduction of real-time quantitative PCR (qPCR) in the early 1990s opened the door for quantitative gene expression analysis.⁴ By incorporating fluorescent dyes or probes during amplification and measuring product accumulation

during the exponential phase of amplification, qPCR provides cycle threshold values that help researchers calculate relative transcript abundance. qPCR's combined sensitivity, specificity, and large dynamic range led to widespread adoption for gene expression studies. Today, qPCR applications range from detecting minimal residual disease in cancer studies to monitoring genetic biomarkers in neurodegenerative conditions such as Alzheimer's disease. Still, qPCR depends on standard curves, allowing only relative quantification, and can be susceptible to variations in amplification efficiency. Digital PCR has emerged as a solution that provides highly sensitive absolute quantification of nucleic acids without the need for standard curves.

Introducing Digital PCR

Scientists developed what we now refer to as digital PCR before qPCR became widely adopted; the concept was first described in the late 1980s as limiting dilution PCR, a method that combined end-point PCR and Poisson statistics to yield an absolute nucleic acid concentration measurement.⁴ In 1999, researchers at Johns Hopkins University coined the term digital PCR to describe this method, whereby a sample is diluted and partitioned to the extent that single template molecules can be amplified individually.

By partitioning a sample into thousands of tiny reactions, each ideally containing zero or one template molecule, digital PCR allows researchers to directly count DNA molecules. After amplification, partitions are classified as positive or negative and statistical modeling provides absolute copy numbers, no longer relying on external standards. [Droplet Digital PCR, or ddPCR](#), represents a refinement of this approach.⁵ ddPCR technology

generates tens of thousands of uniform droplets, dramatically increasing precision and sensitivity. [ddPCR gene expression assays](#) perform exceptionally well for small fold changes, such as expression differences that are 2-fold or lower, detecting low-abundance targets, or analyzing samples that are prone to containing PCR reaction inhibitors.

For translational research workflows, this means detecting subtle gene expression shifts and rare transcripts in limited sample populations, such as circulating tumor cells or patient-derived neurons.

Multiplexing and the Evolution of Gene Expression Analysis

Alongside the evolution of PCR-based gene expression methods, the ability to study multiple genes simultaneously has advanced through multiplexing. Multiplex ddPCR benefits from the partitioning strategy, which minimizes the risk of cross-reactivity or spectral overlap. This enables more reliable detection of multiple transcripts in the same reaction, saving sample and reagents while increasing experimental power.

Taken together, the trajectory of PCR technology shows how innovations have steadily improved sensitivity, precision, and scope. Conventional PCR made detection routine, qPCR turned quantification into a reliable process, and ddPCR has now established itself as the modern-day gold standard for expression analysis. For oncology and neuroscience, where rare transcripts or subtle changes can have substantial biological implications, [ddPCR platforms](#) advance analysis, providing absolute measurements with the robustness required for discovery and clinical research.

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TIPS AND TRICKS ADDRESSING PCR PAIN POINTS

The fields of cell and gene therapy have existed for a long time, first as independent areas of study, and more recently, combined. Throughout this history, a number of key techniques have facilitated cell and gene therapy strategies in the laboratory and the clinic. Today, as technologies such as CRISPR make gene editing and manipulation easier and more accessible, cell and gene therapy is well poised to help shape therapeutics in the 21st century.

PCR-based applications have expanded into diverse fields, including clinical research such as diagnostics and therapeutic development. However, with its increasing implementation in real-world scenarios, the risk of inaccurate or inconsistent qPCR results becomes more pressing.¹ Gene expression tests that are sensitive and specific while also addressing common qPCR pitfalls are an approachable solution for scientists pursuing translational breakthroughs.

Common Challenges in qPCR Workflows

Researchers carefully design and validate primers and probes, meticulously prepare high-quality standards, and include appropriate controls to measure gene expression with precision.¹ Although qPCR is a staple method that has revolutionized gene expression analysis, it requires careful preparation of standard curves, and errors in preparing or quantifying standards can cascade into inaccurate results.

Amplification efficiency is a common culprit behind troubleshooting steps during qPCR workflows, as it

can vary between templates based on factors such as GC content, DNA secondary structures, and the presence of inhibitors in different samples.² These issues become particularly problematic when studying rare transcripts, where low signal can be obscured by background noise. Multiplexing adds another layer of complexity, as designing multiple probes that amplify with similar efficiency and do not interfere with one another requires thorough optimization.¹ Moreover, small differences in reagent batches, thermocycler calibration, or handling practices can cause variability between experiments or laboratories, making reproducibility a persistent pain point.^{1,2}

Strategies and Solutions for PCR Pain Points

By eliminating the need for standard curves and relying instead on absolute quantification through droplet partitioning, [Droplet Digital PCR \(ddPCR\)](#) circumvents one of the most common pain points in qPCR: relative quantification. Even when amplification efficiencies vary within a ddPCR assay, the binary classification of droplets as positive or negative makes the system more tolerant to differences that would confound qPCR analyses. This robustness becomes especially important for low-abundance transcripts, where ddPCR consistently excels in precision and sensitivity.³

Another source of frustration in gene expression analysis is the reproducibility of results across laboratories and over time. Differences in how scientists choose and calibrate qPCR

standards can create inconsistency between data sets that disrupt how gene expression changes are interpreted. In clinically relevant research fields such as cancer or neuroscience, where monitoring changes in gene expression may one day guide therapeutic decisions or provide new diagnostic avenues, reproducibility is particularly essential. Because ddPCR assays produce absolute copy numbers directly, they make comparisons across experiments more reliable.

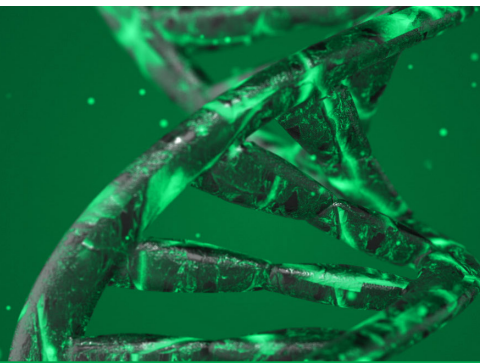
Adopting ddPCR for Flexible Applications

For researchers who are well-versed in performing qPCR assays, incorporating ddPCR into their workflows is straightforward. The amplification reaction is similar, and each ddPCR partition contains the core reagents used in a qPCR reaction to generate a fluorescent signal in response to the presence of a target sequence.^{2,3} Scientists can readily adapt assays from qPCR to ddPCR and achieve consistent and reliable gene expression analysis results.

Ultimately, qPCR remains a powerful and widely used tool, but many of its challenges persist despite best practices. ddPCR directly addresses these limitations with workflows that are approachable for researchers already familiar with qPCR. When ddPCR is paired with carefully designed [assays, validated reagents, and compatible instruments](#), it not only simplifies workflows but also enhances specificity and sensitivity.

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Delving Into Gene Expression Data Analysis and Experimental Reproducibility



Standardized frameworks for designing, executing, and reporting gene expression assays, including qPCR and dPCR, enhance transparency and reproducibility, helping researchers ensure their data is reliable and can be independently verified. The minimum information for publication of quantitative real-time PCR experiments guidelines (MIQE) and digital MIQE guidelines (dMIQE) include detailed checklists and standardized terminology recommendations for qPCR and dPCR experiments and publications.

- ✓ Sample handling
- ✓ Assay design and validation
- ✓ Data analysis

MIQE 2.0 Guideline Focus Points

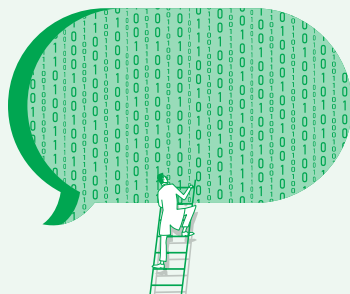
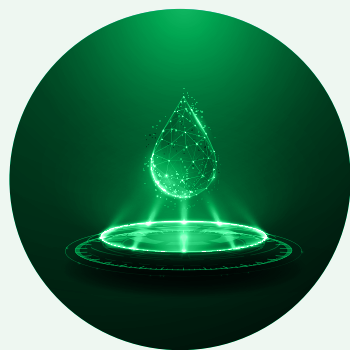
The latest qPCR guidelines, MIQE 2.0, emphasize the importance of efficiency-corrected data analysis, appropriate statistical methods, consistent reporting of technical replicates, and the inclusion of positive and negative controls.¹ They encourage the use of digital resources and data repositories for sharing experimental details, raw fluorescence data, and analysis scripts to foster collaboration, independent result verification, and research output transparency.

The Importance of Normalization

Researchers must account for data analysis variables such as standard curves and reference genes early in their expression assays. Ideally, target standards can be accurately quantified for standard curve consistency, for example by digital PCR.¹ In both qPCR and dPCR gene expression assays, normalization using reference genes supports precise measurements and helps correct technical variability, but inconsistencies in how normalization is performed can introduce additional errors.¹ **ddPCR gene expression probe assays** with relevant and complimentary reference assays help researchers achieve maximum specificity and transcript coverage.

Reliable ddPCR Quantification

Similar to qPCR, specific dMIQE guidelines have been developed to ensure unbiased and reproducible digital PCR experiments, the most recent being dMIQE2020.² These guidelines also emphasize data analysis and provide detailed guidance on calibration, normalization, and controls. Data analysis considerations that can help account for technical variability and accuracy include choosing appropriate partitioning strategies, automated threshold setting, and sharing raw data. **Software programs** that help researchers perform all necessary calibrations and standardizations limit variability and promote consistency, while allowing automatic adjustments that account for reaction efficiency and multiple reference comparisons.



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ddPCR WORKFLOWS FOR GENE EXPRESSION ANALYSIS

Similar to qPCR, ddPCR assays can be preceded by reverse transcription (RT), which converts RNA into complementary DNA (cDNA) and enables gene expression analysis, directly quantifying RNA levels.^{1,2} Unique technical and ease-of-application strengths contribute to RT-ddPCR emerging as an exceptional and flexible tool for RNA quantification.

Two-Step Versus One-Step RT-ddPCR

Two main reverse transcription strategies are available: one-step and two-step RT-ddPCR.³ In a two-step workflow, RNA is first reverse transcribed into cDNA, which can then be stored and used for multiple downstream assays. Following reverse transcription, scientists partition the ddPCR reaction mixture that contains cDNA, primers, probes, and polymerase into tens of thousands of nanoliter-sized droplets. Each droplet functions as an independent reaction chamber for amplification and quantification. This provides flexibility and allows the same cDNA preparation to be interrogated for different transcripts, though it requires more handling and introduces additional opportunities for variability.

In a one-step RT-ddPCR workflow, reverse transcription and PCR amplification occur in the same droplet. This approach streamlines the process, reduces the risk of contamination, and shortens turnaround time, making it particularly useful in clinical or high-throughput research environments.

During thermal cycling for either method, if a droplet contains a target molecule, it will yield a positive fluorescent signal, whereas droplets without template remain negative. Following amplification, researchers analyze the ratio of positives to total droplets via Poisson statistical calculations to derive absolute transcript copy numbers.

The inherent partitioning strategy of any ddPCR workflow, including both one-step and two-step RT-ddPCR methods,

minimizes competition among templates and enables quantifiable and highly sensitive transcript detection. It also contributes to RT-ddPCR quantification being less affected by amplification efficiencies, as poorly amplified partitions can still be detected, thus exhibiting higher tolerance to reaction inhibitors that can hinder researchers' RT-qPCR experiments.⁴

RT-ddPCR Applications

In neuroscience, where researchers often contend with scarce tissue samples and subtle transcript changes, RT-ddPCR offers greater detection power compared to conventional PCR, where the presence of or fluctuations in low-abundance transcripts may be undetectable relative to more abundant housekeeping transcripts. Partitioning allows scientists to analyze each droplet independently, so no signal goes uncounted, offering high accuracy and efficiency.⁵

Researchers have applied RT-ddPCR to analyze serum microRNA levels with potential relevance to neurological conditions such as multiple sclerosis (MS), the progression of which is conventionally studied via markers in cerebrospinal fluid, a sample that is challenging to collect and limited in availability.⁶ RT-ddPCR enabled scientists to validate candidates and measure serum concentration of specific microRNAs with precision, demonstrating higher levels of three transcripts that are significantly linked to progression from relapsing remitting MS to secondary progressive disease.

In translational studies where semi-quantitative hybridization-based methods are often standard, such as in oncology, the ability to reliably quantify slight variations in transcripts poses great promise for improving diagnostic, prognostic, and therapeutic opportunities. For example, in oncology studies, scientists have implemented RT-ddPCR to measure transcripts associated with HER2 expression in human breast tissue samples.³

HER2 is an established therapeutic target in several cancer types, and determining gene expression within tumor samples is a clinical routine that currently relies on a combination of immunohistochemistry (IHC) to evaluate HER2 protein expression and in situ hybridization (ISH) to assess HER2 gene status.⁷

Although this approach allows pathologists to distinguish between HER2 positive and negative tumors with good inter-observer reproducibility and consistency, it is semi-quantitative and may not be similarly robust for classifying emerging clinically relevant subtypes, such as HER2-low cancers.⁸ Researchers have demonstrated that RT-ddPCR can rank order breast cancer samples by HER2 status in agreement with current standard methods, with the added benefit of yielding accurate and precise quantitative gene expression data.³

Simplified and Streamlined Gene Expression Analysis

Beyond technical strengths, RT-ddPCR assays benefit researchers via streamlined experimental processes, simplified workflows, and reduced complexity. Dedicated kits for [one-step reverse transcription, instruments](#) that enable simplified droplet generation workflows, and intuitive [analysis software](#) lower the barrier for adoption. One-step RT-ddPCR also reduces sample handling, leading to faster turn around times. [ddPCR gene expression assays](#) are available as probe or EvaGreen Assays, with protocols for use in one-step or two-step RT-ddPCR protocols.

The removal of standard curves further simplifies data analysis, reducing both workload and potential sources of error. Taken together, the streamlined workflows, reproducibility, and sensitivity of RT-ddPCR make it an indispensable tool for modern gene expression research.

See references on page 7

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