Gene expression analysis

Measuring changes in gene expression with the QuantStudio Absolute Q Digital PCR System

Introduction

Gene expression involves multiple cellular processes, from transcription, processing, and export of mRNA from the nucleus to its translation into protein and posttranslational modification. Each stage is tightly controlled by the cell, and overall expression levels reflect the sum of these steps. Molecular techniques are commonly used to assess gene expression because they are fast, sensitive, and relatively inexpensive. mRNA can be quantified using direct hybridization methods like microarray analysis, RNase protection assays, and northern blotting, or it can be quantified indirectly after reverse transcription and amplification of the resulting complementary DNA (cDNA) by PCR [1].

Quantitative real-time PCR (qPCR) is a variant of PCR that has long been viewed as the gold-standard technique for gene expression studies. qPCR is routinely used to validate individual targets identified with large-scale screening tools, such as microarray analysis and next-generation sequencing (NGS) [2]. qPCR has many advantages over other methods of mRNA quantification, including a wide dynamic range and ease of use. However, although qPCR excels at measuring abundant targets and detecting larger differences in gene expression, it is not always successful at accurately quantifying rare targets or detecting smaller variations in expression levels. qPCR also requires use of an assay with consistent and known efficiency for accurate relative quantification.

Digital PCR (dPCR) is an invaluable tool that can complement qPCR across a wide range of applications, including gene expression analysis. Unlike qPCR, which monitors changes in fluorescence intensity in a single bulk reaction, dPCR divides the reaction into many microreactions. Each reaction is read individually at the end of amplification (endpoint detection) and interpreted as being positive or negative for the target(s) based on the presence or absence of fluorescence. Compartmentalizing the bulk reaction into microreactions allows direct quantification of target sequences through Poisson statistics, which eliminates the need for reference standards and standard curves. Digital PCR excels at highly precise quantification, even when target concentrations are low, and it can often detect small differences more reliably than qPCR. Because dPCR relies on endpoint detection, results are not impacted by variations in amplification efficiency. Therefore, dPCR is generally more robust and reliable for working with challenging samples that may contain PCR inhibitors and low-abundance targets. In contrast, qPCR results are sensitive to variations in amplification efficiency, which can complicate gene expression analysis for certain sample types and low-abundance targets.

Much primary and translational research effort has been devoted to detecting changes in gene expression to elucidate cellular processes and identify potential therapeutic targets for diseases [3-5]. For technical reasons, the focus has historically been on larger differences, generally two-fold or more. However, it has become increasingly clear that changes in mRNA levels of less than two-fold can have a large impact on downstream cellular processes and consequently on disease [6-9]. This is particularly true when the expressed proteins feed into pathways that amplify the initial signal [5], and in the context of multi-transcript gene signatures [10]. Furthermore, complex samples containing multiple cell types or cells at different stages may produce small fold changes in aggregate if a gene is highly upregulated or downregulated in only a subset of constituent cells [11]. Compared to microarrays, NGS, and qPCR, dPCR has excellent precision for detecting changes of less than two-fold, thereby filling a critical gap in gene expression research.

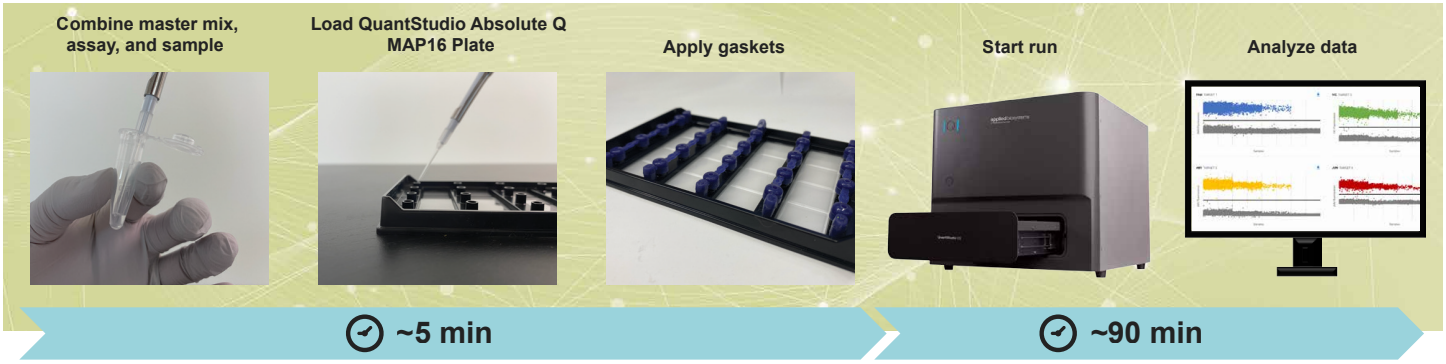
Although dPCR enables absolute quantification, the quality and quantity of RNA input should be considered when comparing different samples. Normalization by mass input is unreliable because it does not reflect factors that can affect the quality of the preparation, such as degradation and the presence of PCR inhibitors. Spectrophotometric methods are not specific for RNA, so the presence of DNA and other contaminants can lead to over-estimation of concentration. Robust gene expression studies require simultaneously amplifying stably expressed mRNAs (also commonly referred to as housekeeping genes), as endogenous controls [12]. By doing so, the expression level of a target can be normalized to the quantity of endogenous control in the same sample to account for sample-to-sample differences in mRNA input and quality. Although singleplex PCR on one or more endogenous controls can be run in parallel in separate reactions from those containing the gene of interest, this approach carries a higher risk of pipetting error. Using multiple endogenous control genes for normalization rather than one is generally advised [13,14], further highlighting the value of multiplexing over singleplexing.

The Applied Biosystems™ QuantStudio™ Absolute Q™ Digital PCR System is an easy-to-use, fast, and flexible dPCR system that enables precise target quantification at low concentrations. The system features a simple workflow with plate setup in as little as 5 minutes and run times of approximately 90 minutes, and no manual transfer of plates between equipment modules is required (Figure 1). The QuantStudio Absolute Q dPCR system also permits multiplexing with multiple dyes, which enables normalization to endogenous controls and measurement of multiple genes of interest in the same reaction.

This technical note describes the performance of the QuantStudio Absolute Q dPCR system for gene expression analysis. We highlight the advantages of dPCR for gene expression analysis, including reliable detection and quantification of changes in low-abundance targets, precise detection of small fold changes, and the ease of performing robust multiplex gene expression analysis.

Materials and methods

cDNA was generated from total human RNA using Invitrogen™ SuperScript™ IV VILO™ Master Mix. All dPCR testing was performed on the QuantStudio Absolute Q dPCR system with Applied Biosystems™ Absolute Q™ Universal DNA Digital PCR Master Mix, using the default thermal cycling conditions. Tests were performed with a multiplex assay containing four catalog Applied Biosystems™ TaqMan™ gene expression assays with the following four dyes: *HPRT1* (FAM-MGB; Hs99999909_m1), *RPLP0* (VIC-MGB; Hs99999902_m1), *GUSB* (ABY-MGB; Hs99999908_ m1), and *POLR2A* (Cy®5-MGB; Hs00172187_m1). Catalog assays can be ordered with custom fluorophores through the **Specialty TaqMan Assay and Oligo Service**. The four assays were present at 1X each in the reaction mix (i.e., all were at 1X—900 nM of each primer and 250 nM of each probe). All qPCR was performed on the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System with the same assays and the same primer and probe concentrations used for dPCR.



Fast and simple workflow—consistent and reliable results

Figure 1. QuantStudio Absolute Q dPCR system workflow. Sample eluates are combined with master mix and the assay(s), then loaded into the Applied Biosystems™ QuantStudio™ Absolute Q™ MAP16 Plate along with isolation buffer. After the gaskets are applied, the plate is inserted into the instrument, and the run is started. Compartmentalization, amplification, and detection occur on the same instrument, requiring no transfer of plates between modules.

Results

Accurate quantification of low-abundance transcripts on the QuantStudio Absolute Q dPCR system

Accuracy and precision are important for detecting changes in mRNA levels. Relative changes in low-abundance transcripts can be difficult to assess because many techniques lack the necessary precision. To compare the precision of dPCR and qPCR at low transcript concentrations, a cDNA sample was 2-fold serially diluted several times, and *POL2RA* and *RPLP0* targets were quantified at each dilution point. A representative one-dimensional dPCR plot is shown in Figure 2A, and representative qPCR amplification curves are shown in Figure 2B. Regardless of the target's concentration, the relative concentration reported by dPCR was consistent with the dilution factor (Figure 2C). The two-fold differences in target concentration between dilutions were readily distinguishable by a clear "staircase" pattern. When tested by qPCR, the same cDNA dilution series showed greater variability at lower concentrations, resulting in less reliable comparisons between sample dilutions (Figure 2D). The variability at low concentrations among technical replicates tested by qPCR made it difficult to discern that the samples were different dilutions. Because dPCR maintains high precision at low concentrations, target quantification and determination of fold changes are more accurate and precise.

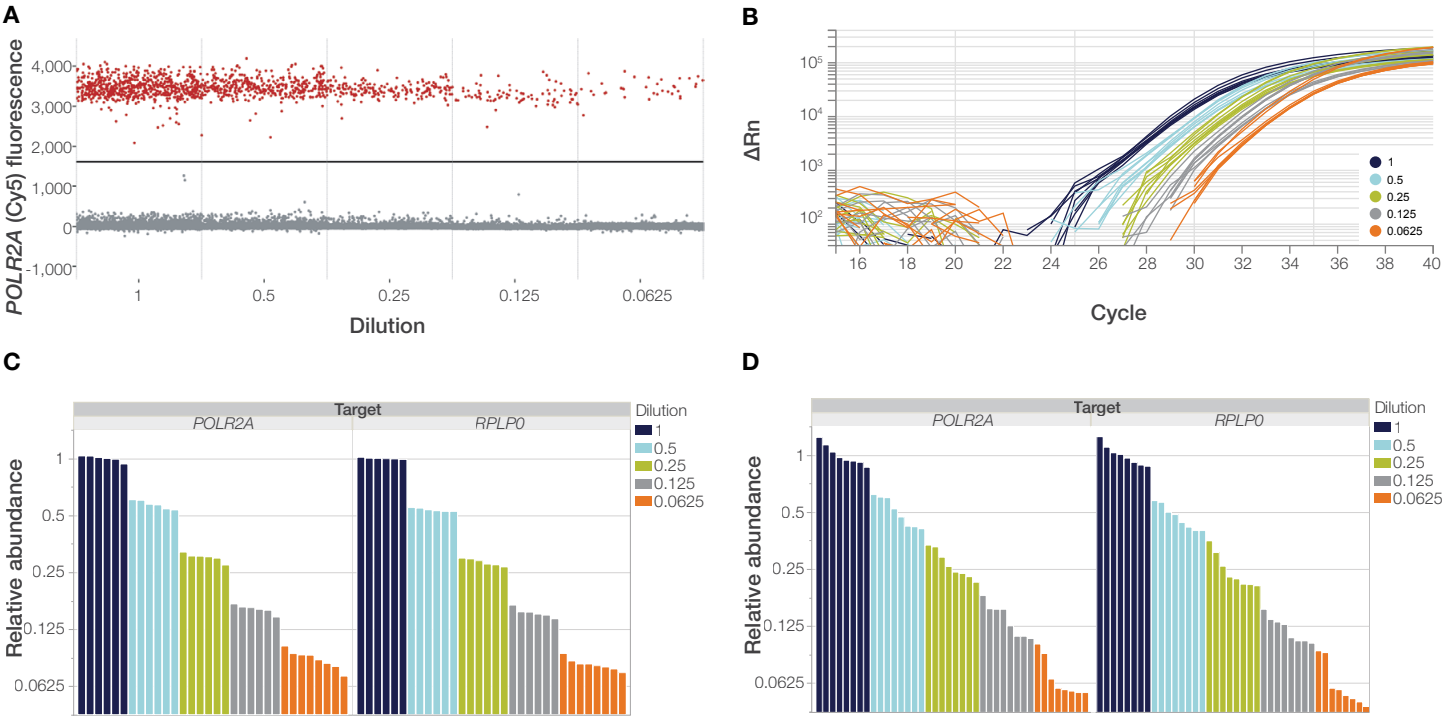


Figure 2. Comparison of dPCR and qPCR for quantifying low-abundance transcripts. To assess variability in quantification after an initial 1:250 dilution of a cDNA sample (dilution 1), the cDNA in subsequent two-fold dilutions was quantified by dPCR and qPCR. **(A)** Representative one-dimensional fluorescence intensity plot for the *POLR2A* target across two-fold dilutions run on the dPCR system. **(B)** Representative qPCR amplification curves for the *POLR2A* target from the highest concentration (dilution 1) to the lowest relative concentration (0.0625 dilution) tested. **(C)** dPCR results showing relative abundance of *POL2RA* and *RPLP0* targets in the dilution series. Each bar represents an individual technical replicate. **(D)** qPCR results showing relative abundance ($2^{\Delta Cq}$) of *POLR2A* and *RPLP0* targets in the dilution series. Each bar represents an individual technical replicate.

Precise quantification of small fold changes

To demonstrate that dPCR could reliably detect small changes in mRNA levels with high precision, cDNA from ten samples, containing characterized small incremental changes in *RPLP0* transcript levels, along with one control, were tested on the QuantStudio Absolute Q dPCR system using the multiplex assay. For this analysis, *RPLP0* expression was normalized to *HPRT1* and *POLR2A*. As shown in Figure 3A, dPCR clearly resolved small fold changes with little variation between replicates. In contrast, qPCR (Figure 3B) produced more variation between replicates, hindering the resolution and identification of small differences between samples.

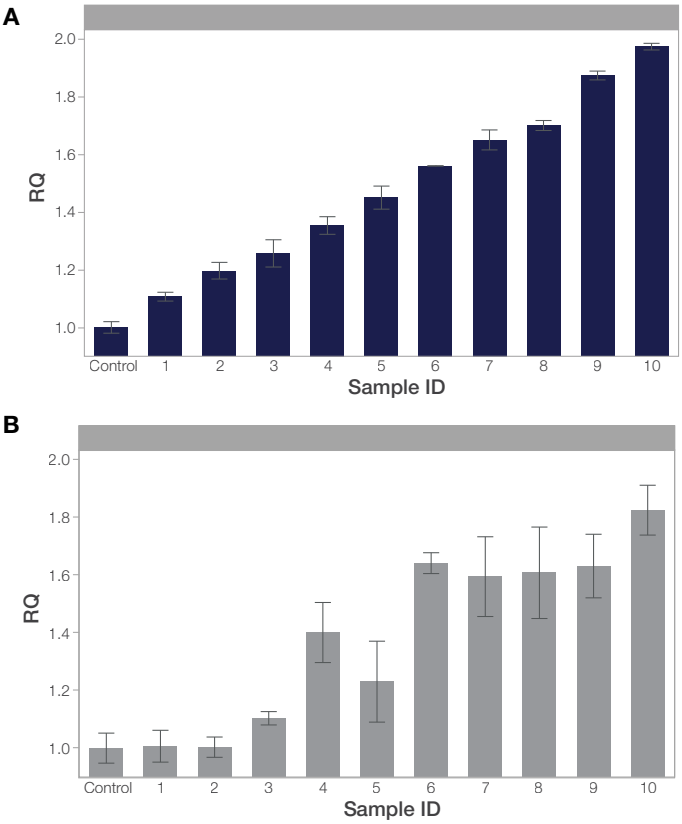


Figure 3. Comparison of dPCR and qPCR for detecting small differences in mRNA levels. Relative quantification (RQ) of cDNA in each sample normalized to the control sample was performed by qPCR and dPCR using the multiplex assay. **(A)** Results obtained with the QuantStudio Absolute Q dPCR system. RQ values were derived by calculating the concentration of the *RPLP0* target in each replicate relative to the values for the *HPRT1* and *POLR2A* endogenous controls in the same well, then expressing that value relative to the mean normalized *RPLP0* target concentration in the control sample. Shown are mean RQ values with error bars representing the standard deviation of three RQ values for that sample. **(B)** Samples tested by qPCR. The $2^{-\Delta\Delta Cq}$ value was determined as follows: the ΔC_q value was first calculated for each *RPLP0* replicate compared with the geometric mean of *HPRT1* and *POLR2A* in the same well. Individual $\Delta\Delta C_q$ values were then determined relative to the mean ΔC_q for the control sample. The $2^{-\Delta\Delta Cq}$ value was then calculated for *RPLP0* in each replicate. The mean of the three $2^{-\Delta\Delta Cq}$ values for each sample is shown, and error bars represent standard deviations. Note that the multiplex assay was shown to produce the same values as the assays run individually by dPCR (Figure 4) and qPCR (data not shown), indicating absence of competition.

Multiplexing made easy

A major benefit of dPCR is the ability to create multiplex gene expression assays with little to no optimization. There is also less concern about competition between assays impacting quantitative results. To demonstrate the ease of dPCR multiplexing, four catalog TaqMan gene expression assays were selected and screened *in silico* for nonspecific binding using the **Request for Multiplexing Support** template. Comparable results were obtained when samples were tested using the 4-plex and singleplex assays (Figure 4). These results demonstrate that dPCR can enable rapid implementation of multiplex assays without the optimization of primer/probe concentrations and cycling conditions often required for qPCR.

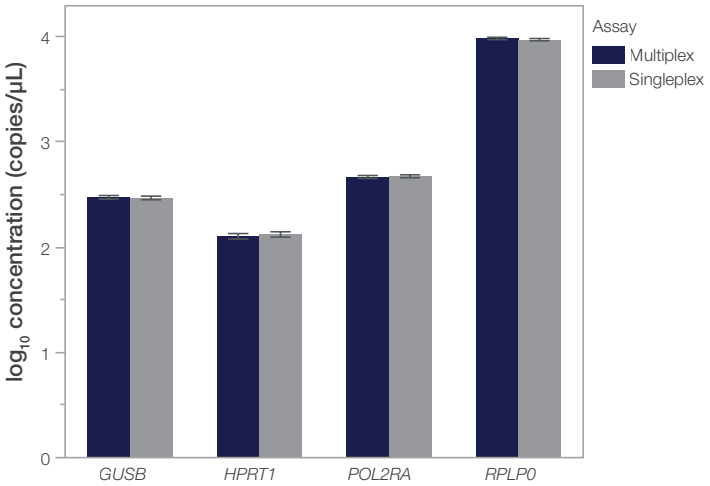


Figure 4. Ease of multiplexing gene expression assays on the QuantStudio Absolute Q dPCR system. Individual assays with the same final primer and probe concentrations used for the multiplex assay were tested in parallel. All testing was performed using the default thermal cycling parameters and the same human cDNA pool. The bars represent $\log_{10}$ of the targets' concentrations determined by dPCR, and the error bars represent 95% confidence intervals.

Conclusion

dPCR and qPCR are complementary techniques for quantifying changes in gene expression. qPCR offers a wider dynamic range, making it well suited for identifying large changes and differences between disparately expressed transcripts. dPCR offers superior precision for detecting small fold changes and low-abundance transcripts, whether you are in the discovery phase or confirming results obtained with qPCR or other methods. The QuantStudio Absolute Q dPCR system is a highly robust dPCR platform with a simple workflow, quick turnaround time, and multiplexing capability for multiple dyes.

References

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Related products and services

Description	
TaqMan predesigned assays	thermofisher.com/taqman
Multiplexing support service	thermofisher.com/multiplex-assay-support
Order predesigned assays with non-catalog dye/quencher combinations such as ABY-MGB and Cy5-MGB	thermofisher.com/taqman-custom-assay-oligo

Ordering information

Description	Quantity	Cat. No.
Absolute Q Universal DNA Digital PCR Master Mix (5X)	200 reactions	A72710
QuantStudio Absolute Q MAP16 Plate Kit	12 plates	A52865
QuantStudio Absolute Q Digital PCR System, desktop	1 system	A52864
QuantStudio Absolute Q Digital PCR System, desktop, extended warranty, SmartStart orientation	1 system	A53267
SuperScript IV VILO Master Mix	50 reactions	11756050
	500 reactions	11756500
TaqMan Gene Expression Assay *HPRT1*		FAM-MGB; Hs99999909_m1
TaqMan Gene Expression Assay *RPLP0*		VIC-MGB; Hs99999902_m1
TaqMan Gene Expression Assay *GUSB*		ABY-MGB*; Hs99999908_m1
TaqMan Gene Expression Assay *POLR2A*		Cy5-MGB*; Hs00172187_m1

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