Application Note

Same target, different answer? Why assay selection matters differently in qPCR than dPCR

Franziska Brodkorb, Christina Engel, David Bauer, Ronny Kellner

QIAGEN GmbH, Hilden, Germany

Introduction

The design of a PCR assay plays a critical role in the accuracy and reliability of target quantification. Multiple assays can be developed to detect the same target of interest and differences in primer and probe sequences, amplicon length, melting temperature (T_m), GC content and other sequence characteristics can lead to substantial differences in amplification efficiency and overall assay performance (Figure 1).

In quantitative PCR (qPCR), these variations can introduce systematic bias, as differences in amplification efficiency directly affect the calculation of target abundance. Consequently, alternative assay designs for the same target may produce significantly different relative quantification results, impacting data interpretation and comparability across studies.

Digital PCR (dPCR), in contrast, relies on endpoint detection of individual partitioned reactions and provides absolute quantification without the need for standard curves. Because quantification is based on counting positive and negative partitions rather than amplification kinetics, dPCR is less susceptible to differences in assay amplification efficiency. ▷

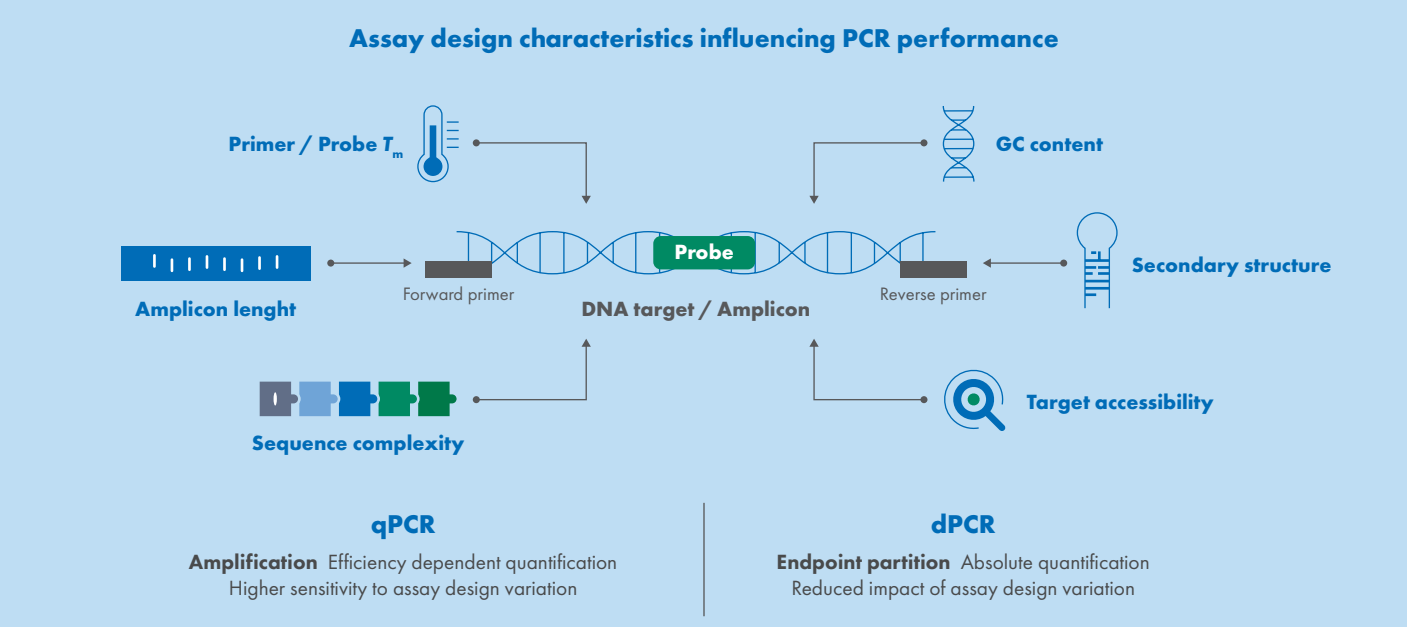


Figure 1. Assay design characteristics that can influence PCR assay performance. Key design parameters, including primer and probe melting temperature (T_m), GC content, amplicon length, secondary structure, sequence complexity and target accessibility, can affect amplification efficiency and overall assay performance. Differences in these characteristics among assays targeting the same sequence can lead to variability in qPCR quantification, but their impact on dPCR is reduced due to endpoint-based analysis.

In this application note, we evaluate qPCR's limitation of producing variable results when using alternative assay designs. Four representative targets were selected and for each target two independent assay designs were developed. By comparing the quantification results obtained with these alternative assay designs across both qPCR and dPCR, we assess the extent to which assay choice influences measured target abundance.
We also demonstrate how dPCR can provide more robust quantification that is less affected by differences in assay designs.

Materials and methods

Workflows and experimental setups

Gene expression analysis was performed using a two-step RT-PCR workflow. cDNA was first generated and subsequently used as a template for probe-based qPCR and dPCR reactions. Reactions were set up as duplex assays and performed in quadruplicates, with each reference gene assay design analyzed alongside the *IFNG* assay. This setup enabled direct comparison of normalized *IFNG* expression measurements obtained with alternative assay designs for the reference gene.

cDNA synthesis

cDNA was synthesized from QIAGEN Human XpressRef Universal Total RNA (cat. no. 338112) using the QuantaBio® qScript™ cDNA SuperMix (cat. no. 95048-025), following the manufacturer's instructions. The resulting cDNA was diluted in water prior to use and served as a template for all qPCR and dPCR reactions, providing equivalent cDNA inputs for comparisons between assay designs and PCR technologies. cDNA input amounts were adjusted according to the respective reaction format, with cDNA corresponding to 1 ng RNA used in 12 µL dPCR reactions and cDNA corresponding to 1.67 ng RNA used in 20 µL qPCR reactions.

Assays

A total of eight probe-based reference gene assays were used, consisting of two independent assay design versions for each of four reference genes: *ARFGEF2* (ADP-ribosylation factor guanine nucleotide-exchange factor 2), *RPL32* (ribosomal protein L32), *SDHA* (succinate dehydrogenase complex flavoprotein subunit A), and *HPRT1* (hypoxanthine phosphoribosyltransferase 1). These reference genes were selected because of their stable expression across a broad range of tissue types.

All assay designs follow a common set of design principles, with amplicon GC content ranging from 45% to 66%, amplicon lengths between 72 and 350 bp and probe-to-primer melting temperature (T_m) differences of 4 to 8°C. Each reference assay design was combined with the *IFNG* (interferon gamma) assay in a separate 2-plex reaction. This resulted in eight individual 2-plex assay combinations, allowing assessment of the effect of alternative reference gene designs on normalized *IFNG* expression by both qPCR and dPCR.

qPCR and dPCR analysis

All dPCR tests were performed on the QIAcuity® nanoplate digital PCR system using 96-well 8.5K nanoplates and the QIAcuity High Multiplex Probe PCR Kit (cat. no. 250133). qPCR tests were performed on the Bio-Rad® CFX Opus™ 96. For qPCR, 96-well plates were used together with a multiplex qPCR master mix from supplier A. qPCR runs and cycling protocols were performed according to the manufacturer's instructions. qPCR results were analyzed with the OPUS Software and dPCR results with the QIAcuity Software Suite v3.2. For each 2-plex reaction, *IFNG* expression was normalized to the corresponding reference gene assay design. In qPCR, efficiency-corrected target quantities were determined in accordance with MIQE guidelines (1). Raw fluorescence versus cycles data without baseline correction were used to determine reaction- and target-specific amplification efficiencies and efficiency-corrected target quantities (N_0) using RDML tools (Ref. 2; https//www.gear-genomics.com).

Non-baseline-corrected fluorescence data were imported into the software, which performs baseline correction for each reaction individually, identifies the window of linearity within the exponential phase of amplification and applies linear regression to the selected data points. The amplification efficiency of each reaction was derived from the slope of the resulting regression line, which was extrapolated back to cycle 0 to provide a relative target quantity, N0, expressed in fluorescence units.

In dPCR, copy number concentrations were obtained directly from endpoint partition analysis. For both qPCR and dPCR, the ratios of target quantities were then used to calculate normalized expression values (1).

Design-dependent effects were assessed by comparing normalized *IFNG* expression values obtained with the two assay design versions of each reference gene. For each method, deviations between reference gene design versions were calculated relative to the corresponding comparison design and expressed as percentage change.

Percentage deviation was calculated as follows:
% deviation = (((normalized *IFNG* expression with reference gene design version 2 – normalized *IFNG* expression with reference gene design version 1)/normalized *IFNG* expression with reference gene design version 1) x 100)-1.

$$\text{\% deviation} = \left(\frac{\text{normalized } \textit{IFNG} \text{ expression with design 2} - \text{normalized } \textit{IFNG} \text{ expression with design 1}}{\text{normalized } \textit{IFNG} \text{ expression with design 1}} \right) \times 100$$

Results and discussion

Digital PCR minimizes the impact of assay design on target quantification

To investigate the impact of different assay designs on target quantification, relative abundances for *IFNG* were calculated using four different reference genes in two independent assay designs. Results obtained by dPCR and qPCR were compared to assess how resistant each method was to variations in assay design.

Reference-gene design agreement was markedly lower for qPCR than for dPCR (Figure 2). In qPCR, the absolute design discrepancy reached 53.6% for *RPL32* (1.54-fold; p=0.017) and 25.4% for *SDHA* (1.25-fold; p=0.009), demonstrating substantial design-dependent variation. *ARFGEF2* showed a smaller but still noticeable discrepancy of 11.5% (0.89-fold; p=0.096). These findings highlight a key limitation of qPCR: the selected reference-gene assay design can substantially alter the calculated expression level of the target gene.

By contrast, dPCR produced substantially more consistent normalized *IFNG* expression values. The absolute design discrepancies were only 5.0% for *ARFGEF2*, 1.1% for *SDHA*, and 4.8% for *HPRT1*, with no significant design effects noted for these genes. *RPL32* showed a 7.8% dPCR discrepancy (0.92-fold; p=0.0102). Thus, dPCR was more resistant to reference-gene design variation and even the largest dPCR discrepancy remained limited compared to qPCR.

The stronger design-related variation observed with qPCR is consistent with the method's dependence on amplification efficiency for quantification. Even when assay designs target the same gene, differences between primers and probes can alter amplification kinetics, thereby affecting inferred target abundances. This can either directly or indirectly impact relative quantification depending on whether the assay targets the gene of interest or a normalizing reference gene. Because normalized expression is calculated relative to reference gene(s), these errors can propagate into the final gene expression estimate.

dPCR was less affected by these design-dependent differences as target quantification is based on endpoint partition counting rather than real-time amplification ▷

kinetics. As a result, minor differences in amplification efficiency or reaction kinetics have a reduced impact on the final copy number estimates, provided that amplification is sufficient to resolve target-containing partitions from the background signal level. This represents a key advantage of dPCR over qPCR: normalized expression results are more invariant to the particular assay designs used, reducing the risk that technical assay-design effects are misinterpreted as biological differences.

Overall, Figure 2 demonstrates that alternative assay designs can lead to different normalized gene expression results, with qPCR showing larger absolute discrepancies and therefore greater susceptibility to design-dependent bias. In contrast, dPCR showed greater consistency across assay design variants, demonstrating the strength of endpoint-based absolute quantification in minimizing the impact of assay design on normalized gene expression analysis.

This improved robustness makes dPCR particularly advantageous when assay redesign, target accessibility or sequence-dependent amplification differences cannot be fully controlled, as dPCR supports more comparable and reliable measurements than qPCR.

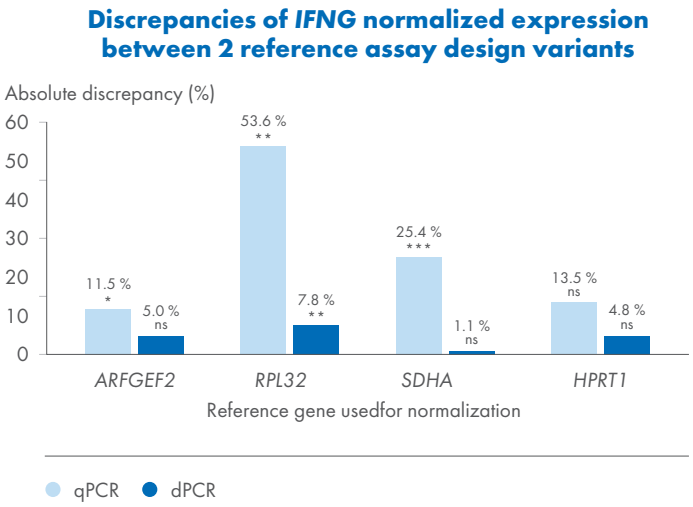


Figure 2. Normalized *IFNG* expression is less affected by variations in reference gene design in dPCR than qPCR. Reference-gene design discrepancy in normalized *IFNG* expression across four reference genes (*ARFGEF2*, *RPL32*, *SDHA*, and *HPRT1*) measured by qPCR and dPCR. Bars show the absolute percentage difference between Design 1 and Design 2 for each reference gene for qPCR (light blue) and dPCR (dark blue). qPCR and dPCR results are based on four replicates per design. P-values were calculated using two-sided Welch t-tests. Significance symbols are defined as *** p<0.01, ** p<0.05, * p<0.10, and ns p≥0.10. Larger qPCR discrepancies for all four reference genes indicate lower agreement between reference-gene designs than observed with dPCR.

Table 1. qPCR shows greater assay-design sensitivity, whereas dPCR provides more robust normalized *IFNG* quantification

Reference gene	qPCR – design-sensitive					dPCR – design-robust					Interpretation
	Fold change	Difference	95% CI	p-value	Magnitude	Fold change	Difference	95% CI	p-value	Magnitude	
ARFGEF2	0.885	**11.5%**	0.785–0.985	0.0964	Small	1.050	**5.0%**	0.956–1.144	0.3355	Negligible	Larger design discrepancy in qPCR
RPL32	1.536	**53.6%**	1.285–1.787	0.0167	**Large**	0.922	**7.8%**	0.892–0.952	0.0102	Negligible	
SDHA	1.254	**25.4%**	1.159–1.349	0.0094	**Moderate**	1.011	**1.1%**	0.931–1.092	0.7988	Negligible	
HPRT1	1.135	**13.5%**	0.945–1.324	0.1904	Small	1.048	**4.8%**	0.894–1.203	0.5609	Negligible	

Fold change represents Design 2 relative to Design 1, whereas discrepancy is the absolute percentage deviation from unity. Approximate 95% confidence intervals were calculated by propagated uncertainty and p-values were obtained using two-sided Welch t-tests. Discrepancy magnitude was classified as negligible (<10%), small (10–20%), moderate (20–50%) or large (>50%).

Conclusion

In conclusion, these results demonstrate that alternative assay designs for the same gene target can substantially influence normalized gene expression results, particularly when using qPCR. Because qPCR quantification depends on amplification kinetics and efficiency correction, design-related differences in primer and probe performance can propagate into the normalized target expression value, leading to inaccurate conclusions and reduced comparability between experiments.

In contrast, dPCR shows greater consistency across reference gene design versions, supporting more robust normalization and reducing the likelihood that technical assay design effects are interpreted as biological variation. By relying on endpoint partition-based absolute quantification, dPCR minimizes the impact of design-dependent amplification differences and enables superior comparability between experiments, even when different assay designs are used.

Therefore, dPCR provides a clear advantage over qPCR for gene expression studies, benefiting from reliable normalization, reproducible quantification and consistent comparison of results across assay designs and experimental runs.

References

1. Bustin SA, et al. MIQE 2.0: Revision of the minimum information for publication of quantitative real-time PCR experiments guidelines. *Clin Chem.* 2025;71(6):634–651.
2. Untergasser A, Ruijter J, Benes V, van den Hoff MJB. Web-based LinRegPCR: Application for the visualization and analysis of (RT)-qPCR amplification and melting data. *BMC Bioinformatics.* 2021;22(1):398.

Ordering Information

Product	Contents	Cat. no.
QIAcuity One, Four or Eight Platform System	Digital PCR instrument for detecting up to 5 fluorescent dyes or up to 8 fluorescent dyes operating under software version 3.1 or higher, enabling up to 12-plex analysis	911021, 911042, 911052
QIAcuity High Multiplex Probe PCR Kit	1 mL 4x concentrated QIAcuity High Multiplex Probe PCR Master Mix, 2 x 1.9 mL RNase-free Water, QN Internal Control DNA	250133, 250134
QuantaBio qScript™ cDNA SuperMix	For 25, 100 or 500 reactions: 5x concentrated reagent (reaction buffer, recombinant ribonuclease inhibitor (RIP), qScript reverse transcriptase, random hexamer and oligo(dT) primer, enzyme stabilizers, performance-enhancing additives	95048-025
QIAcuity Nanoplate 8.5k 96-Well	10 QIAcuity Nanoplate 8.5k 96-well, 11 Nanoplate Seals	250021

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