Application Note

Transferring and optimizing assays from quantitative PCR to digital PCR on the QIAcuity® system

Introduction

The most common nucleic acid detection and quantification approach is real-time quantitative PCR (qPCR). However, digital PCR (dPCR), in its various formats (chips, droplets, etc.), is a rapidly evolving technology in this area. The two techniques share similarities and differences, and it is due to those differences, several applications benefit from dPCR over qPCR. This includes partitioning the samples into thousands of individual reactions, absolute quantification without standard curves and the reduced susceptibility to PCR inhibitors. Digital PCR follows end-point amplification, and each partition can be positive or negative, depending on the presence or absence of a target sequence counted by a fluorescence measurement, resulting in a binary readout. Poisson statistics is then applied to determine the absolute quantity of target DNA in a sample. Moreover, the end-point measurement enables quantification independent of the amplification efficiency, thereby allowing dPCR to be used for low-abundance target quantification even in complex samples or highly precise quantification of very low DNA copy numbers.

Quantitative PCR is a well-established technique with optimized protocols in most molecular biology laboratories, so when establishing digital PCR for the first time or migrating existing assays from qPCR to dPCR, different aspects need to be considered. These include, but are not limited to, primer and probe concentrations and cycling conditions (for example, time, temperature and cycle number). However, note that the fundamental detection chemistries and assay formats of dPCR (for example, LNA probes, hydrolysis probes and DNA-binding dyes, primer design, one-step or two-step reaction, etc.) will seem highly familiar to an existing qPCR user (Figures 1, 2). Here we demonstrate how to optimize your assays on a microfluidic nanoplate-based digital PCR system, the QIAcuity, and provide recommendations for a seamless transfer. Moreover, the QIAcuity dPCR workflow is very similar to qPCR.

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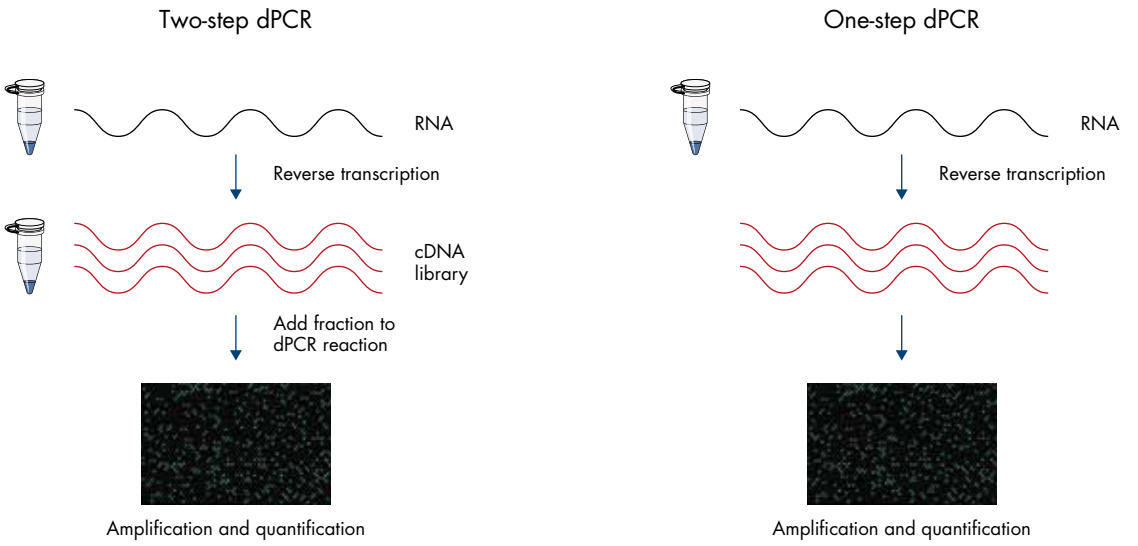


Figure 1. Familiar assay formats for dPCR: one-step reaction (left) and two-step reaction (right). cDNA synthesis from RNA input and PCR amplification of cDNA is performed in the same reaction well in one-step dPCR. cDNA is synthesized from RNA in bulk, and PCR amplification is done on a sub-sample of the cDNA in two-step dPCR.

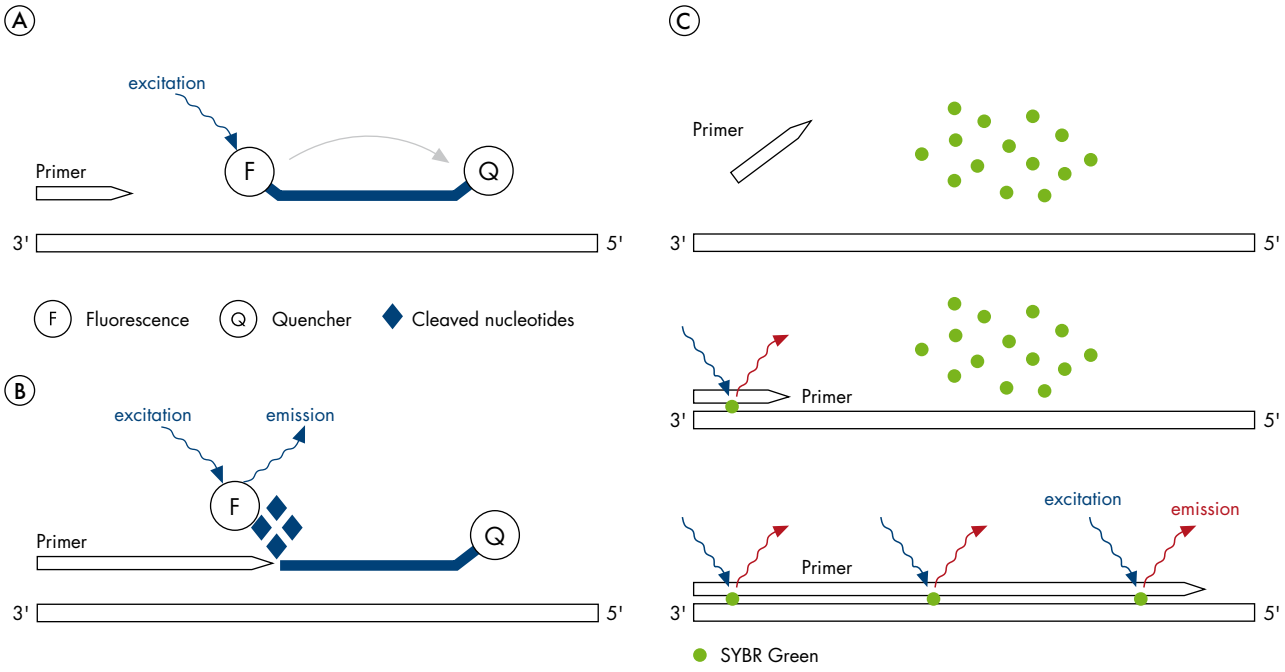


Figure 2. Familiar assay and detection chemistry options: working principle of hydrolysis probes (left) and DNA-binding dyes (right). Three oligos (two PCR primers and one detection probe with fluorophore and quencher) are shown on the left. Primers and probe anneal to target sequence during thermal cycling. When intact, the probe will have minimal fluorescence. The polymerase extends from primer, encounters bound probe and hydrolyzes. Cleavage of the fluorophore from the distal quencher activates the fluorescent signal. This signal is proportional to the amount of accumulated PCR product. On the right, two oligos (forward and reverse PCR primers) and a sequence-agnostic dsDNA binding dye (e.g., EvaGreen) are shown. The PCR primers anneal to the target sequence during the PCR annealing step. Taq DNA polymerase extends the primer during the PCR extension step, and EvaGreen binds to the newly formed double-stranded DNA. Upon DNA binding, a conformational change of the dye is induced, and it starts to fluoresce brightly. This signal is proportional to the amount of accumulated PCR product.

Standardization in qPCR came at a high cost of irreproducible data. In 2009, a group of qPCR experts published the MIQE guidelines for reproducible experiments[1]. Those guidelines shaped present-day qPCR, which is considered a gold standard technique in gene expression[2]. Fast forward, dPCR also took advantage of the publication of the dMIQE guidelines[3] to ensure global standardization.

The best practices of assay design and optimization provided here also ensure adhering to the latest dMIQE guidelines[4] for standardizing experimental protocols, maximizing efficient utilization of resources and further enhancing the impact of this powerful technology.

Conversion strategy and considerations

When no assay is available in hand but there is a need for high resolution target quantification, our general recommendation is as follows:

- Obtain pre-designed and conditionally validated assays from commercial sources – these are usually dMIQE-compliant and tailored to unique master mixes and kinetics of the thermocyclers specific to a system
- Published, peer-reviewed designs – review literature for assays and assess dMIQE compliance. While this practice is common in the research community, we advise checking for the specificity of the published primers.
- Design in-house assays following the recommended conditions mentioned below for the QIAcuity

Regardless of the starting point – no prior assay, established qPCR assay or established dPCR assay on another platform, ensure meeting the following primer and probe design criteria when using the QIAcuity.

Primers

- Use specialized design software (e.g., Primer3Plus or Primer Express)
- Amplicon ideally ≤150 bp
- 18–30 nucleotides in length with 30–70% GC
- T_m of the primers should be 58–62°C and within 2°C of each other
- Avoid
  - Highly repetitive sequences
  - 3'-end cross-complementarity
  - Within or across primer complementarity
  - 3' template mismatch
  - ≥3 Gs or Cs at 3' end
  - Regions with secondary structure specifically at the binding sites of the primers
- Ensure that primer sequences are unique for your template sequence with bioinformatics (e.g., BLAST search)

Probes

- Use specialized design software (e.g., Primer3Plus or Primer Express)
- The T_m of probes should be 8–10°C higher than the T_m of the primers
- Avoid a G at the 5'-end of probes and runs of ≥4 G nucleotides
- Choose the binding strand so that the probe has more C than G bases
- Ensure that primers and probes are not complementary to each other
- Design using the same settings to ensure that they will work optimally under the same cycling conditions (60°C annealing/extension)

It is important that the fluorescent label attached to the probe is compatible with one of the detection channels of the QIAcuity instrument (Table 1).

Table 1. Fluorophore compatibility

Channel	Excitation(nm)	Emission (nm)	Example fluorophores
Green	463–503	518–548	FAM
Yellow	514–535	550–564	HEX, VIC
Orange	543–565	580–606	TAMRA, ATTO 550
Red	570–596	611–653	ROX, Texas Red
Crimson	590–640	654–692	Cy5

It is also worth considering the following reaction (primer-probe concentrations) and thermal cycling conditions for the respective QIAcuity PCR Mix for best results (Tables 2, 3).

Table 2. Recommended reaction conditions

	Volume/reaction		
Component	Nanoplate 8.5k (24-well, 96-well)	Nanoplate 26k (24-well)	Final concentration
4x Probe PCR Master Mix	3 µl	10 µl	1x
10x primer–probe mix 1*	1.2 µl†	4 µl†	0.8 µM forward primer 0.8 µM reverse primer 0.4 µM probe
10x primer–probe mix 2, 3, 4, 5* (for multiplex)	1.2 µl†	4 µl†	0.8 µM forward primer 0.8 µM reverse primer 0.4 µM probe
Restriction Enzyme (optional)	Up to 1 µl	Up to 1 µl	0.025–0.25 U/µl
RNase-free water	Variable	Variable	
Template DNA or cDNA (added at step 4)	Variable‡	Variable‡	
Total reaction volume	**12 µl**	**40 µl**	

* For respective dye recommendation for the probe and available channels on the QIAcuity, please see the QIAcuity User Manual or the *QIAcuity User Manual Extension: Application Guide*.

† Volume might vary, depending on concentration of the primer/probe mix used.

‡ Appropriate template amount depends on various parameters. Please see the *QIAcuity User Manual Extension: Application Guide* for details.

Table 3. Recommended thermal cycling conditions

Step	Time	Temperature (°C)
PCR initial heat activation	2 min	95
2-step cycling (40 cycles)		
Denaturation	15 s	95
Combined annealing/extension	30 s	60*

* Temperature during annealing/extension and number of cycles might vary depending on assay type.

Whether following the recommended assay conditions for the QIAcuity or those used on your current platform during migration, it is advisable to perform pilot runs to determine further optimization.

Pilot runs should be performed by:

- Including a well-characterized positive control, negative control and a no template control (NTC)
- Modifying thermal cycling parameters outside of annealing/extension step, or the hot start if required
- Creating template dilutions to ensure staying within the dynamic range
- Running a dPCR reaction on the QIAcuity using the recommended and your currently used concentration of assay components
- Assessing their initial performance (Figure 3 A–B)

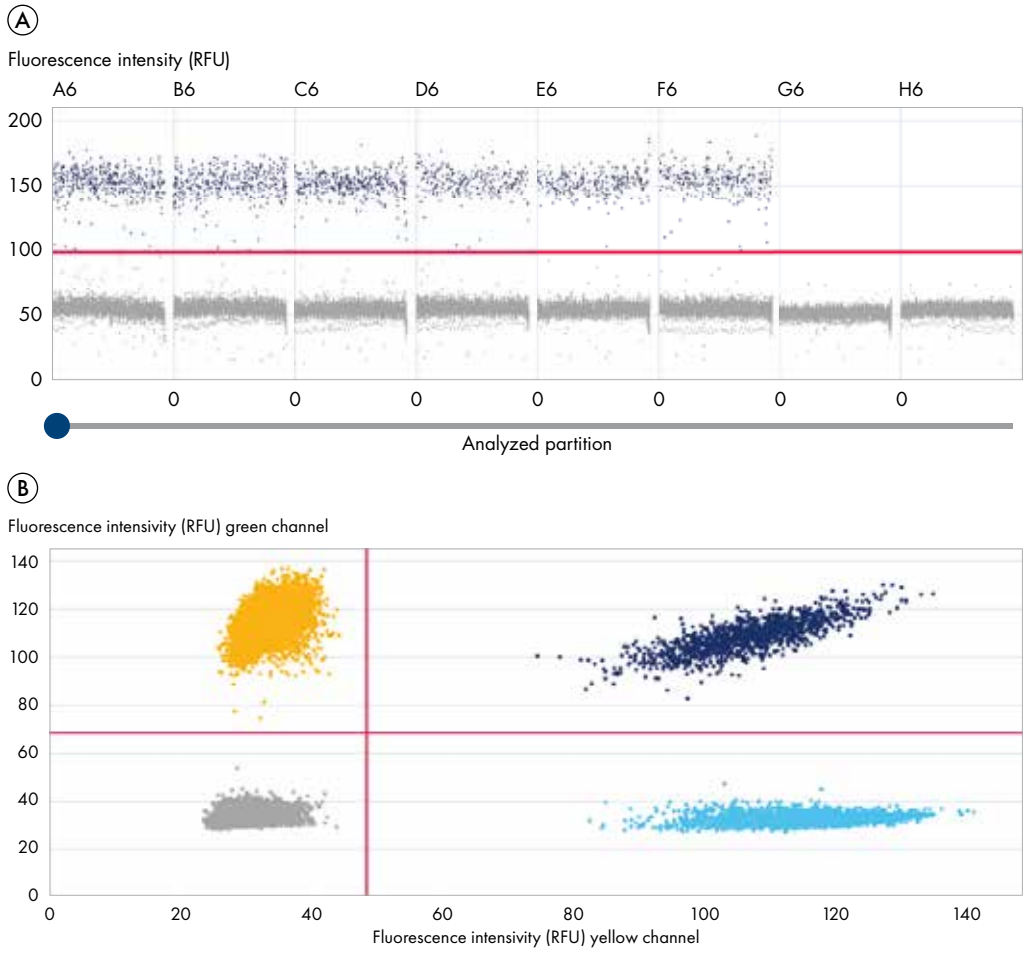


Figure 3. Representative (A)1D and (B) 2D data evaluating the initial performance of a dPCR assay. Criteria for 1D data include the presence of two populations (positive and negative), sufficient signal:noise ratio, meaning high RFU of positive population, low RFU of negative population and the ability to identify the threshold between two populations, and clean NTCs. Criteria for 2D data obtained from a dPCR LNA mutation detection/BRAF V600E assay include the presence of four populations (single positives, dual positives and dual negatives), sufficient signal:noise ratio, meaning high RFU of positive populations, low RFU of negative populations and the ability to identify the threshold between four populations, and clean NTCs.

dPCR assay optimization

Should the performance be suboptimal, optimizing the following parameters can help separate the positive and negative partition signals better, improve PCR efficiency, increase probe specificity and selectivity, reduce assay artifacts and resolve intermediate partition signal.

1. Modifying annealing temperature, depending on the use of dye or probe (Figure 4 A–B)
   Even well-working temperature conditions established for other qPCR and dPCR reagents might be suboptimal since primer and probe annealing properties strongly depend on the composition of the master mixes

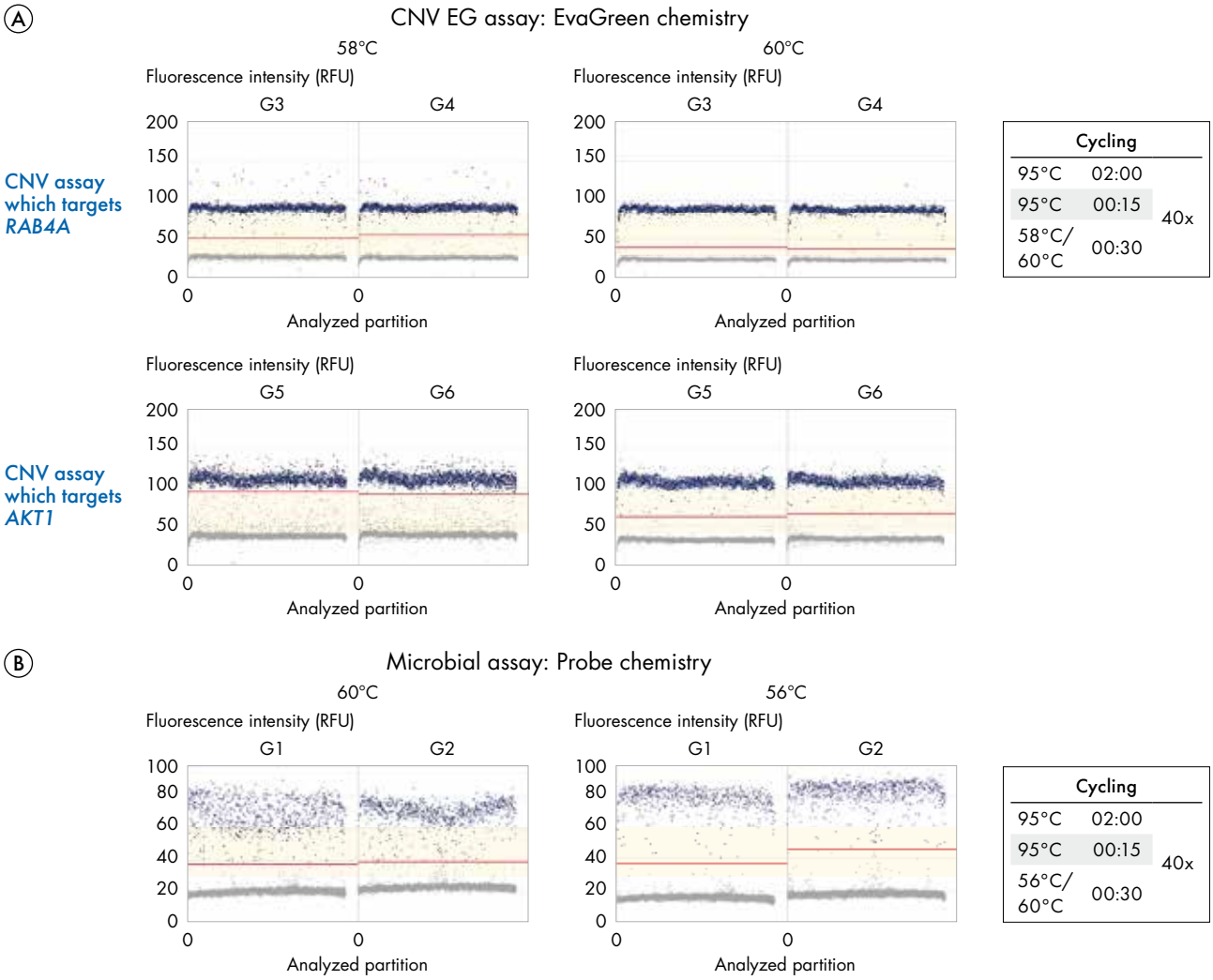


Figure 4. Annealing temperature change resolves intermediate partitions. A dPCR Copy Number Assays targeting RAB4A and AKT1 were run using wild-type gDNA samples as the template and QIAcuity EG PCR Master Mix. 4 ng of the sample was loaded per reaction, per condition in QIAcuity Nanoplate 8.5K, 96-well. Standard QIAcuity instrument and imaging setup were used. The adaptation in annealing temperature resulted in a reduction of rain and an increase in the signal-to-noise ratio. **B** A single-plex Microbial DNA qPCR Assay targeting Lactobacillus plantarum was run using Microbial DNA Positive Control V2 (Cat. Nr. 338135) as the template and QIAcuity Probe PCR Master Mix. Samples were run in duplicates per condition in QIAcuity Nanoplate 8.5K, 96-well. Standard QIAcuity instrument and imaging setup were used. The adaptation in annealing temperature resulted in a reduction of rain.

2. Modifying annealing/extension time, depending on the use of dye (30 seconds) or probe (60 seconds) (Figure 5).

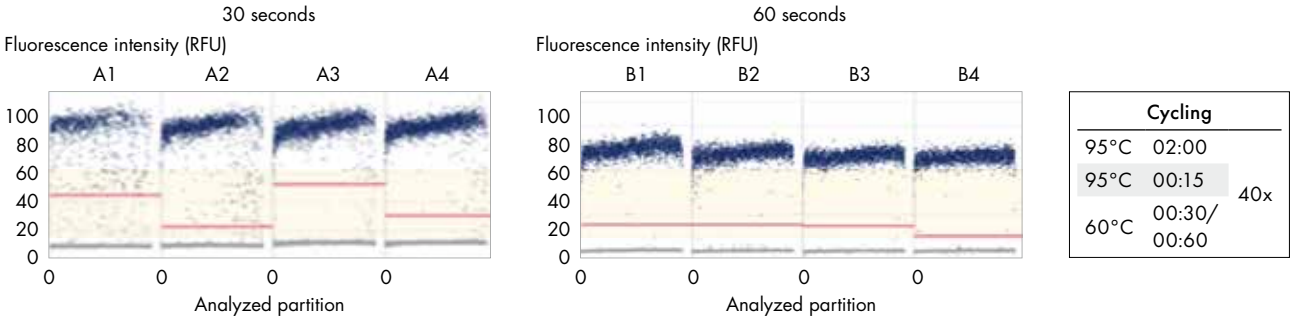


Figure 5. Annealing/extension time change resolves intermediate partitions. Digital PCR reactions were performed on the QIAcuity system in QIAcuity Nanoplate 8.5K, 96-well using a TaqMan® PCR (hydrolysis probe PCR) assay targeting the single-copy human ERBB2 gene from genomic DNA. The cycling parameters used are as indicated in the table. Standard QIAcuity instrument and imaging setup were used. The increase in annealing/extension time resulted in a reduction of rain and an increase in the signal-to-noise ratio.

3. Performing a temperature gradient to determine the optimal annealing temperature

   Optimal annealing temperatures can be easily determined by a quick temperature gradient experiment on any real-time cycler since all QIAcuity kits are compatible with any real-time cycler. Adhere to the recommended cycling conditions outlined in the respective manuals.

4. Performing restriction enzyme digestion to improve template accessibility

5. Adapting the primer-probe concentration (Figure 6)

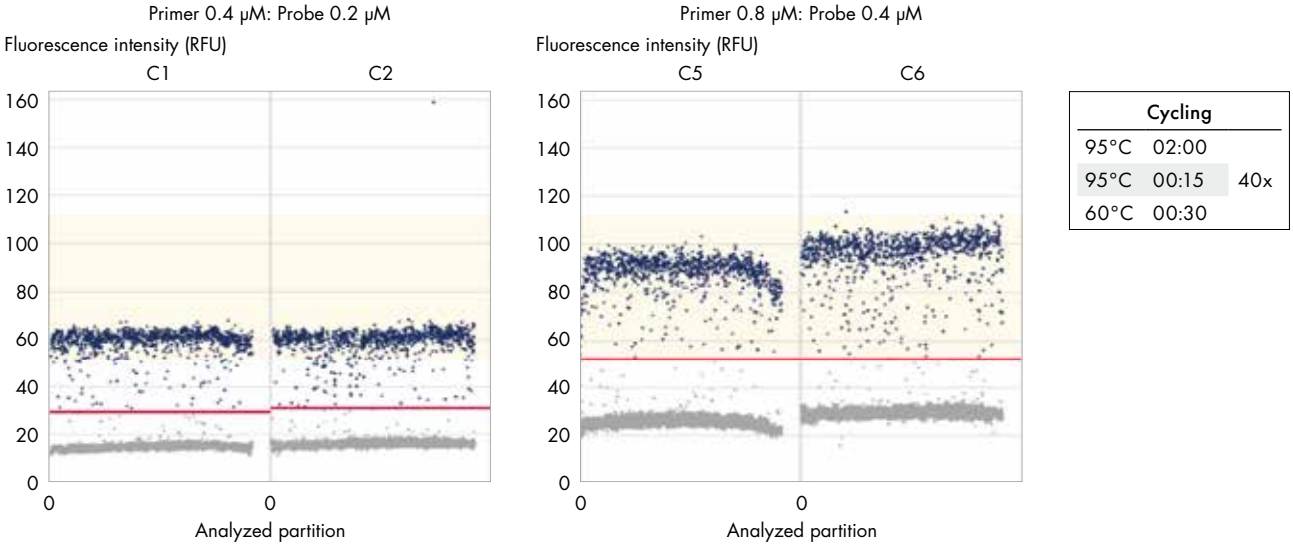
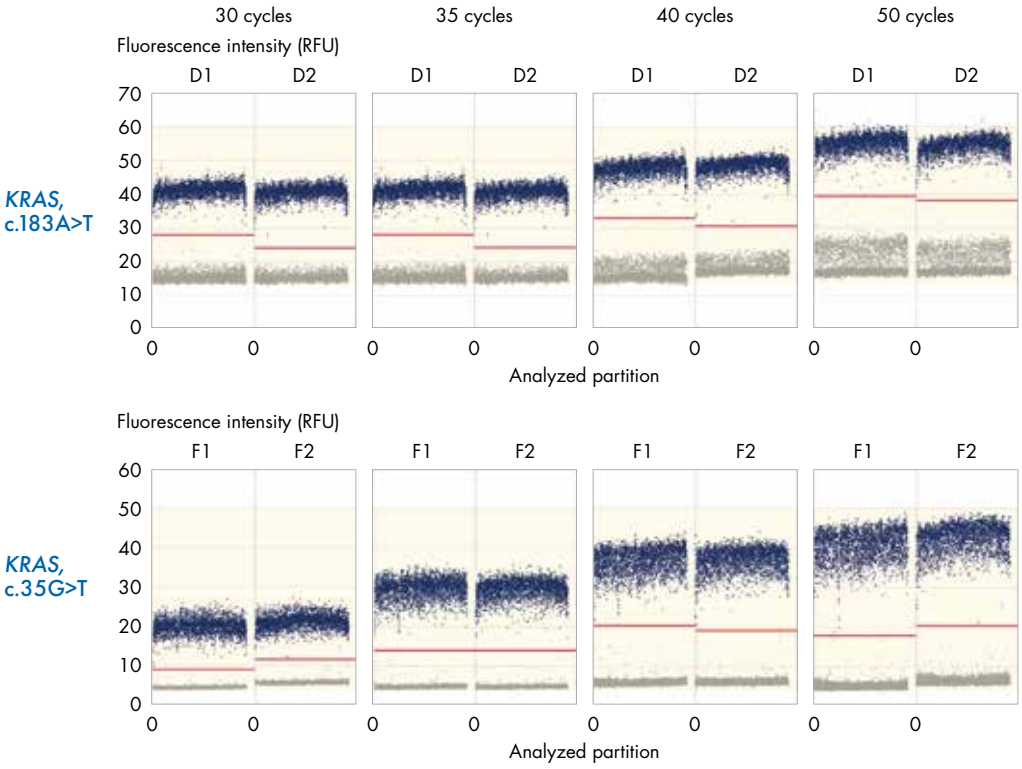


Figure 6. Primer-probe optimization increases positive partition signal. A single-plex Microbial DNA qPCR Assay targeting *Faecalibacterium prausnitzii* was run using Microbial DNA Positive Control V2 (Cat. Nr. 338135) as the template and QIAcuity Probe PCR Master Mix. Samples were run in duplicates per condition in QIAcuity Nanoplate 8.5K, 96-well. Standard QIAcuity instrument and imaging setup were used. The adaptation resulted in better separation of positive and negative partitions, increasing positive partition signal.

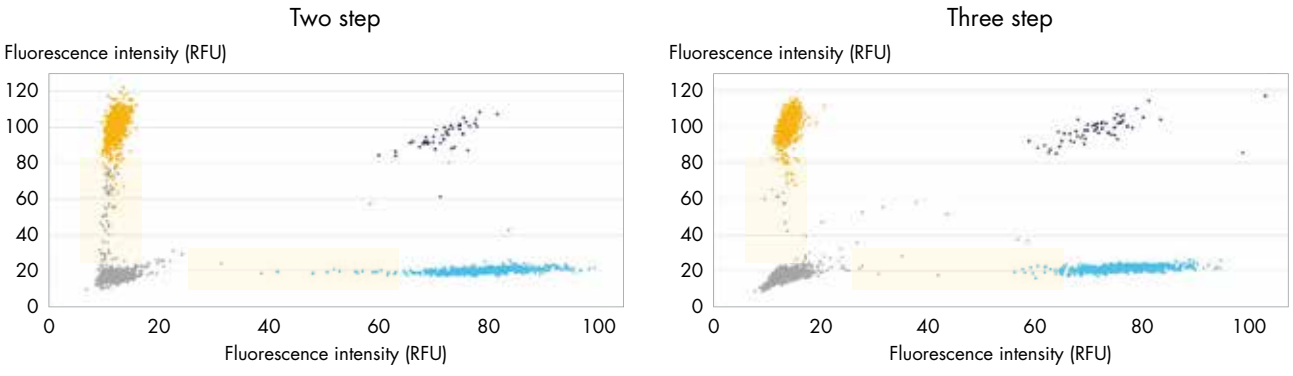
6. Modifying cycle numbers, increasing from 40 to 50 or even 60 (Figure 7)



Cycling		
95°C	02:00	1x
95°C	00:15	30x, 35x, 40x, 50x
60°C	00:30	

Figure 7. Cycle number optimization can increase separation. Digital PCR reactions were performed on the QIAcuity system in QIAcuity Nanoplate 26K, 24-well using LNA mutation assays specific for KRAS mutations. KRAS-specific duplex mutation assay. Wild-type gDNA at 1000 copies/µl was used as the sample in duplicates. Standard cycling and imaging conditions were used. The positive populations detected by the HEX-labeled WT probe in the Yellow channel are shown here. The adaptation increased positive signal, thus demonstrating better separation of positive and negative partitions.

7. Converting 2-step cycling into 3-step cycling with separate steps for annealing and extension at optimal temperatures (Figure 8)

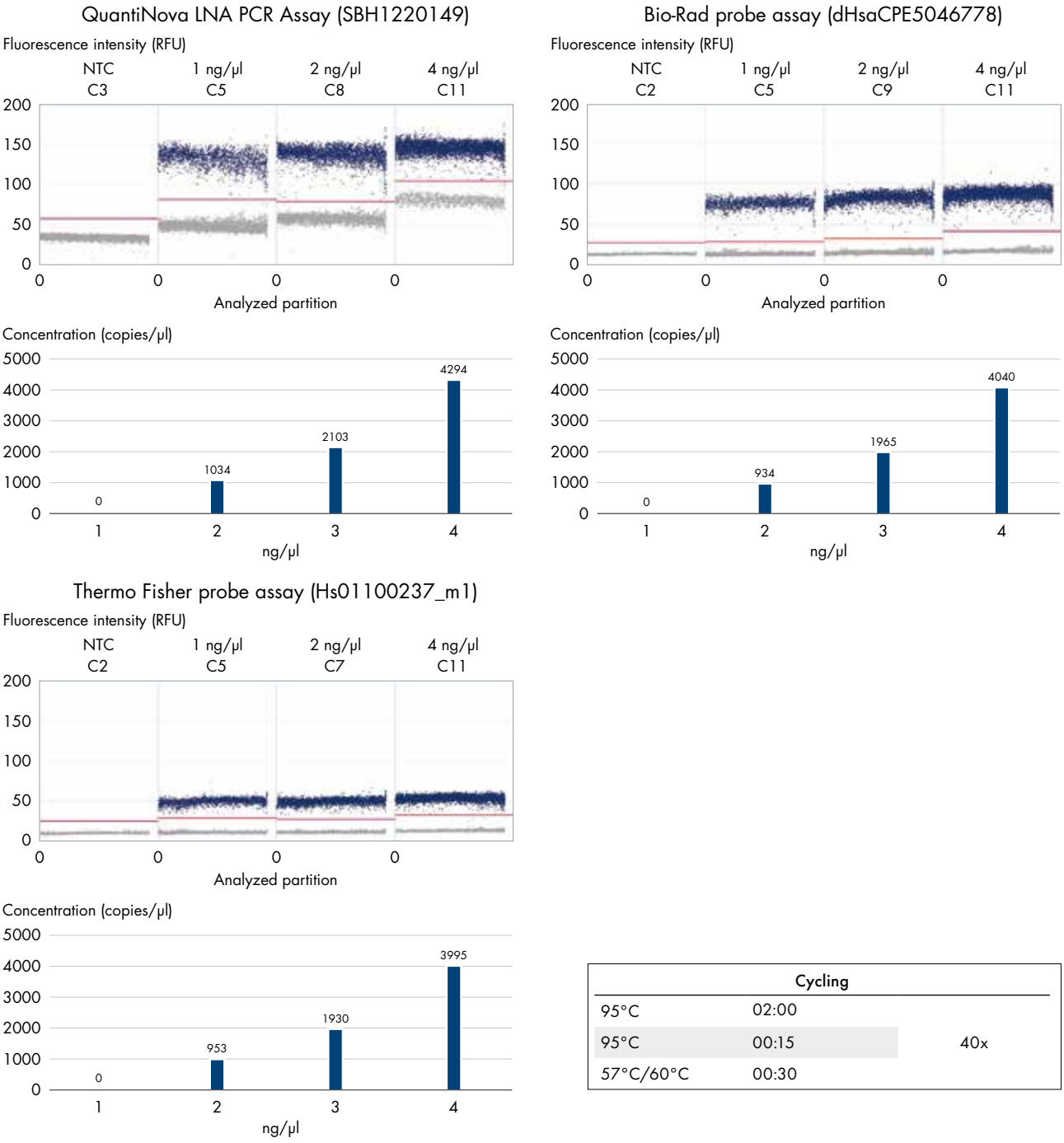


Cycling		
95°C	02:00	
95°C	00:15	40x
60°C	00:30	

Cycling		
95°C	02:00	
95°C	00:15	40x
60°C	00:15	
72°C	00:15	

Figure 8. Transition from two- to three-step thermal cycling can improve separation. 100 copies/µl wild-type gDNA samples were tested with CDH1 (FAM)/TERT (HEX) in a duplex reaction. QIAcuity Probe PCR Master Mix was used. The cycling parameters are as indicated in the table, and the standard QIAcuity imaging setup was used. This adaptation improves the cluster separation.

Commercial assays may perform well with minor modifications (Figure 9). Commercial, premixed real-time PCR assays often come with final concentrations of primer and probes, deviating from the recommendations for the QIAcuity mixes. Nonetheless, without further adaptation, they might work with the concentration recommended by the assay supplier. If results using the 1x concentration are not satisfactory, adapt the concentration of the commercial assay to the recommended primer-probe concentration for the respective QIAcuity mix. This is easily achievable for probe-containing assays with a primer:probe ratio of 2:1. If other primer-probe ratios are used for the assay, adapt the concentration of the assay so that it will have the primer concentration recommended for the respective QIAcuity mix.



Cycling		
95°C	02:00	
95°C	00:15	40x
57°C/60°C	00:30	

Figure 9. Commercial assays may perform well with minor modifications. This example assay compared the QuantiNova® LNA PCR Assay for KAT8 with probe-based assays for the same target gene from Bio-Rad® and Thermo Fisher®, revealing very similar quantification results. Three individual gene expression assays run in QIAcuity Nanoplate 8.5K, 96-well on a QIAcuity dPCR instrument. cDNA template was prepared from UHR (universal human reference RNA from Thermo Fisher Scientific) using the QuantiTect RT Kit (Cat. Nr. 205313). QuantiNova LNA PCR Assay used the QIAcuity EG PCR Kit. The Bio-Rad and Thermo Fisher probe assays used the QIAcuity Probe PCR Kit (Cat. Nr. 250102). 1D Scatterplot depicts one representative of triplicates for each concentration. The numbers above bars show mean concentrations of 3 replicates. Each well was loaded with a 12 µl sample containing the template concentration. Standard QIAcuity instrument and imaging setup were used. The experiment demonstrates the easy adaptation of commercial assays to QIAcuity nanoplate dPCR.

Conclusion

A combination of experimental factors and processes should be considered when migrating home-brew or commercial qPCR and dPCR assays from other suppliers into dPCR assays on the QIAcuity, making that transition highly robust. A summary of the recommendations is provided here.

- Ensure assay design meets criteria described in *QIAcuity User Manual Extension: Application Guide* at **www.qiagen.com/HB-2839**
- Follow recommended conditions for respective QIAcuity PCR Mix
- Check the compatibility of fluorophores
- Perform pilot tests and assess their initial performance prior to further optimization

Ultimately, a successful transition to digital PCR can overcome interlaboratory variations in precision and accuracy and lead to global standardization.

References

1. Bustin, S.A. et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 55(4):611-22.
2. Abdel Nour, A.M. et al. (2014) Five years MIQE guidelines: the case of the Arabian countries. PLoS One. 9(2):e88266.
3. Huggett, J.F. et al. (2013) The digital MIQE guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. Clin Chem. 59(6):892-902.
4. dMIQE Group, Huggett, J.F. (2020) The Digital MIQE Guidelines Update: Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020. Clin Chem. 66(8):1012-1029.

To learn more about QIAcuity nanoplate digital PCR, visit:
www.qiagen.com/dPCR

The QIAcuity is intended for molecular biology applications. This product is not intended for the diagnosis, prevention or treatment of a disease.

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