Digital PCR

Running existing assays on the QuantStudio Absolute Q Digital PCR System

Key takeaways:

- **Robust data**—sophisticated quality control metrics, including in-run background subtraction and AI-powered microchamber evaluation, deliver reliable and accurate target quantification
- **Convenient workflow**—sample preparation and plate setup similar to real-time PCR yield a familiar and simple workflow with a single user touchpoint
- **Seamless assay transition**—workflow and instrumentation compatible with standard real-time PCR chemistry enable easy adoption of digital PCR testing into labs
- **Multi-target analysis**—four optical channels for detecting targets to increase the amount of information per reaction
- **Consistent fluidic loading**—consistent microchamber loading minimizes variation between and within runs to enable precise and reliable results

Introduction

Digital PCR (dPCR) utilizes the same biochemistry as real-time PCR (qPCR) to amplify and detect target sequences. As a general principle, existing well-designed qPCR and dPCR assays can be transitioned to the Applied Biosystems™ QuantStudio™ Absolute Q™ Digital PCR System with minimal optimization. This guide answers some common questions related to transitioning existing assays and provides general information to help evaluate assay performance within dPCR.

There are two key differences between dPCR and qPCR (Figure 1). First, the dPCR reaction volume is initially divided into thousands of isolated microreactions prior to thermal cycling. In this scenario, individual target sequences are colocalized with no, or very few, competing PCR targets. This reduced PCR competition facilitates multiplexing of quantitative assays. It also enables high sensitivity for rare-target detection among related sequences, such as quantification of <0.1% variant allele frequencies for single-nucleotide variants.

The second key difference between qPCR and dPCR is the use of endpoint measurement for target quantification. After thermal cycling is complete, the fluorescence signal is measured to distinguish microreactions as either positive or negative for the target sequence. Use of an endpoint measurement alleviates the dependence on PCR assay efficiency, unlike qPCR measurements for which changes in assay efficiency can impact results. Furthermore, the binary analysis of the endpoint data provides direct determination of target quantification without any dependence on standard curves or calibration with reference materials.

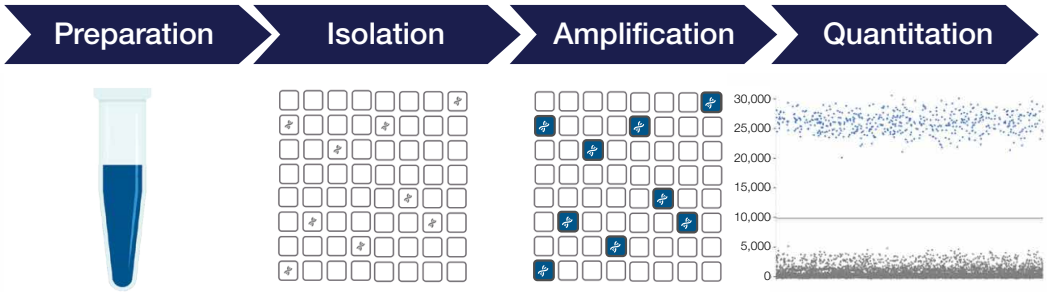

Figure 1. Overview of digital PCR workflow. For the QuantStudio Absolute Q system, preparation of the dPCR reaction mix is nearly identical to that of the qPCR reaction mix. Two key differences between dPCR and qPCR are: (i) the isolation of the bulk reaction mix into thousands of separate microreactions, and (ii) endpoint-based detection that classifies microreactions as positive or negative for the target, enabling direct target quantification through the application of Poisson statistics. For the QuantStudio Absolute Q system, the instrument automatically isolates the reaction mix into microchambers, performs thermal cycling, and collects fluorescence signals, all without any intervention by users between steps.

Quality control

The Applied Biosystems™ QuantStudio™ Absolute Q™ Analysis Software automatically applies a series of quality control (QC) checks to the collected dPCR data and removes QC-rejected microchambers from the final analysis.

- **Microchamber filling:** Applied Biosystems™ Absolute Q™ Digital PCR master mixes contain a loading dye that enables the system to omit microchambers with incomplete filling from the analysis. This QC check helps eliminate false-negative microchambers from biasing the concentration determination.
- **Background subtraction:** For every dPCR run, the system captures pre-PCR background signal for each individual array on the plate. This background signal is subtracted from the endpoint measurement, removing autofluorescence or non-PCR related artifacts and reducing the potential for false-positive microchambers.
- **Microchamber QC:** The raw data are evaluated by an artificial intelligence (AI)-powered algorithm that identifies and rejects problematic microchambers based on the fluorescence signal pattern observed within individual microchamber images. This QC check provides further protection against false positives by rejecting microchambers with signals that are not representative of true PCR fluorescence.

This collection of quality control mechanisms helps reduce the potential for false positives and false negatives to provide reliable answers. Protection against false positives is critical for assessing low-abundance targets for which the presence of a small number of positives can significantly impact data interpretation. The removal of false negatives helps reduce the risk of inaccurate quantification results.

Applied Biosystems™ QuantStudio™ Absolute Q™ MAP16 Plates provide consistent microchamber loading, enabling precise and reproducible dPCR results. Figure 2 shows how the total number of microchambers accepted for analysis is consistently very close to the nominal 20,480 chambers per array. In dPCR, "dead volume" refers to the amount of the dPCR mix that is not used in the final data analysis. Digital PCR systems that yield inconsistent numbers of microreactions can complicate reproducibility of results and lead to higher dead volumes. Higher dead volumes can impact the accuracy of assays that require a low detection limit, since target molecules will have a lower chance of actually being detected in the analysis. For the QuantStudio Absolute Q system, each sample array of a MAP16 plate should generally produce around 20,000 QC-passed microchambers. This consistent microchamber filling, along with the plate's microfluidics design, yields a very low dead volume (<5%), resulting in extremely efficient reagent use and analysis of over 95% of the loaded sample.

The total count of QC-passed microchambers is displayed by the QuantStudio Absolute Q dPCR Analysis Software. A lower count of QC-passed microchambers generally indicates a problematic run; this is often resolved by performing the recommended spin of the dPCR mix prior to loading into the plate (discussed in a subsequent section).

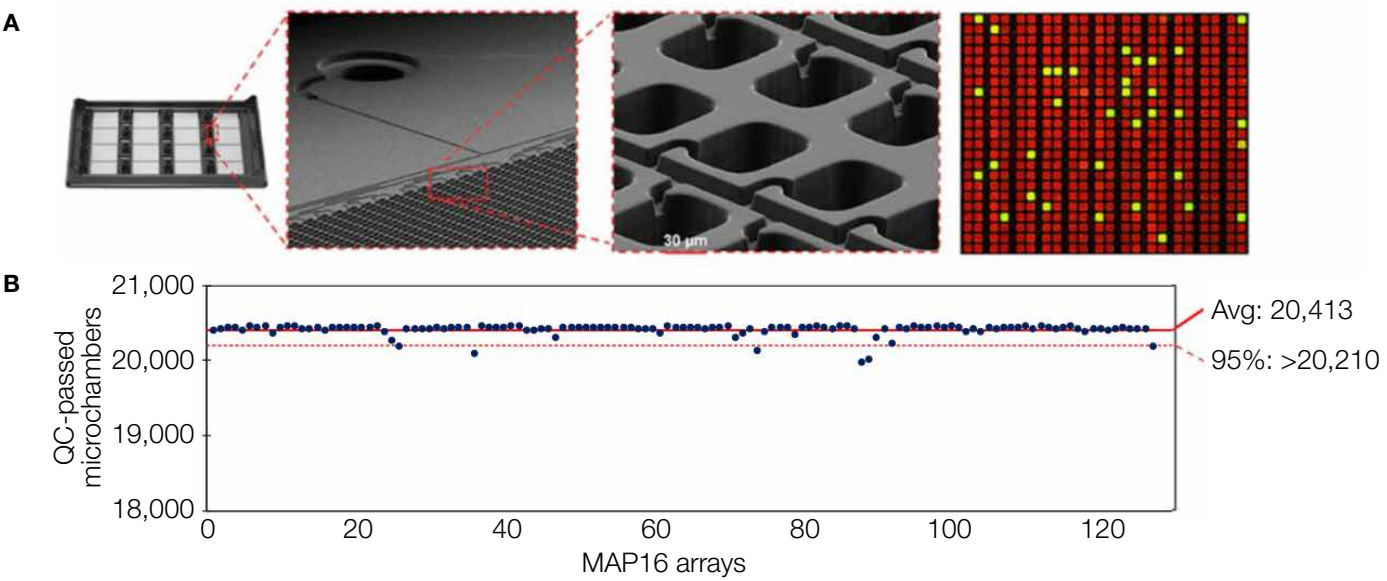

Figure 2. Microfluidic array plate (MAP) technology enables consistent creation of dPCR microreactions. (A) Each sample loading port on the plate is connected to 20,480 microchambers that create the isolated reactions for dPCR. **(B)** The total number of QC-passed microchambers from each array from eight QuantStudio Absolute Q MAP16 plates. On average, there were 20,413 microchambers per array included in the final data analysis, with 95% of arrays having over 20,210 QC-passed microchambers.

Assays

Within the final dPCR reaction mix, we recommend a primer concentration of 900 nM and a probe concentration of 250 nM for each assay. These are the standard concentrations for predesigned Applied Biosystems™ TaqMan™ Assays and are recommended for both our dPCR and qPCR systems. Lower primer or probe concentrations may produce suboptimal dPCR results. If multiplexing assays together for dPCR, "primer limitation" should generally not be used because the division of the sample into isolated microreactions limits competition between targets. Single-quenched probes provide optimum performance for the QuantStudio Absolute Q system. The instrument performs background subtraction on a per-sample basis, which compensates for imperfect quenching of fluorescence signal from the intact probes.

Thermo Fisher Scientific offers a catalog of predesigned TaqMan Assays. When used with the Applied Biosystems™ Absolute Q™ Universal DNA Digital PCR Master Mix, all predesigned TaqMan Assays are backed by the TaqMan Assay Performance Guarantee.* To search our catalog of predesigned assays, visit **thermofisher.com/taqman**.

Fluorophores

The QuantStudio Absolute Q system has five optical channels and is factory calibrated for Applied Biosystems™ FAM™, VIC™, HEX™, ABY™, and JUN™ reporter dyes, and Cytiva™ Cy®5 reporter dye. One of the five optical channels is dedicated to the Applied Biosystems™ ROX™ QC dye that is present in all Applied Biosystems™ Absolute Q™ master mixes. For the blue and yellow channels, the system supports the FAM and ABY dyes, respectively. The green channel supports the VIC and HEX dyes, and the dark red channel supports the JUN and Cy5 dyes. Only one dye per channel can be used per assay (i.e., VIC or HEX dye, JUN or Cy5 dye); this enables multiplexing with up to four reporter dyes. FAM and VIC dyes are often used for single and duplex assays, while ABY and Cy5 dyes are commonly added for assays combining third and fourth targets for multiplexing.

Compared to DNA-binding dyes such as Invitrogen™ SYBR™ Green I dye, probes provide an added layer of specificity to an assay, minimizing signal generation from off-target sequences and nonspecific primer-dimer generation. Some primer sets may work adequately in the presence of their target sequence but generate nonspecific signals in the absence of their target (for example, a primer-dimer signal within no-template controls). For target quantification, dPCR requires some microreactions to not contain the target sequence. Analogous to the potential nonspecific amplification in a no-template control, primer pairs may generate signals from nonspecific amplification in microreactions absent of the target sequence. For these reasons, use of TaqMan probe-based chemistry is generally encouraged for dPCR experiments. However, if use of a DNA-binding dye assay is desired, Applied Biosystems™ Absolute Q™ DNA Digital PCR master mixes can be supplemented with Invitrogen™ SYBR™ Green I Nucleic Acid Gel Stain (Cat. No. S7563) to a final concentration of 0.5X in the dPCR reaction mix. When performing an assay with SYBR Green I dye, the blue channel should be used for data collection with the other optical channels disabled.

Template amount

To calculate a target concentration when performing dPCR, it is required that some microreactions do not contain the target sequence, limiting the maximum amount of target that can be loaded. This requirement leads to a reduced dynamic range in dPCR compared to qPCR. Samples that produce C_q values less than ~15–20 are likely to contain target concentrations above the dPCR range (a specific C_q cutoff value would be specific to the assay and qPCR instrument). Poisson statistics reveal that the best measurement precision in dPCR occurs at a concentration of ~1.6 copies of target per microreaction, corresponding to ~3,700 copies/µL within the QuantStudio Absolute Q MAP16 dPCR plate. The ideal quantity of target to load may be higher or lower depending on the experimental needs and application type. When performing rare-target detection, for example, it may be beneficial to load higher amounts of the wild type sequence to achieve the desired detection sensitivity of the low-abundance rare mutant target.

For dPCR, the quantity directly measured is the concentration of target copies present, often reported in **copies per microliter** (cp/µL). If an experiment is focused on **quantity** of nucleic acid, such as nanograms per reaction, the working range based on mass will vary by sample type. For example, 1 ng of human genomic DNA corresponds to roughly 300 copies of haploid genome. However, 1 ng of plasmid or a synthetic DNA sequence could represent millions of target copies. Online calculators like ours at **thermofisher.com/dna-calculator** can be used to help determine an appropriate template input quantity.

Template digestion

To obtain accurate quantification of tandemly linked target sequences with dPCR, the nucleic acid molecule must be broken between the target sequences to allow their independent distribution into microreactions—otherwise, results will undercount the target concentrations. Restriction digestion or fragmentation of the template may be necessary if performing copy number variation analysis, or when quantifying targets that are present more than once on a given nucleic acid molecule.

* Terms and conditions apply. To see full details of the guarantee, go to **thermofisher.com/taqmanguarantee**.

Physical fragmentation can be used for this purpose if the sequences are distantly separated on the nucleic acid molecule. However, restriction enzyme digestion offers a more controlled and reproducible approach. The enzyme used must have cut sites between the linked target sequences but not cut within the amplicon of the assays. Restriction enzymes can be added directly to the dPCR reaction to provide a convenient and rapid workflow. Additional incubation time of the reaction mix with the restriction enzyme may or may not be needed, depending on the sample and enzyme used. Previous experiments have demonstrated sufficient sample DNA digestion without adding incubation time to the standard plate setup process.

Reaction mix preparation

Preparing the Absolute Q dPCR reaction is similar to preparing a qPCR reaction (Table 1). After the dPCR reaction components are combined, it is important to mix by pipetting or vortexing to ensure a homogeneous solution, which is crucial for random distribution of the target sequences into the microreactions. Before transfer into the QuantStudio Absolute Q MAP16 plate, the dPCR reaction mix should be centrifuged to separate any physical debris that may interfere with microfluidic reagent distribution. If the nucleic acid extract is suspected of containing particulates or debris from the original sample, it may help to also centrifuge the nucleic acid prior to adding it to the dPCR reaction mix.

Thermal cycling

The thermal cycling conditions for assays should generally remain the same or similar across platforms. For example, if optimized cycling temperatures for an assay were previously determined by qPCR, those conditions can serve as a starting point when transferring to the QuantStudio Absolute Q system.

Otherwise, assay evaluation can start with the thermal cycling recommendations provided by the user documentation for the specific Absolute Q dPCR master mix used. In addition to cycling temperatures, the cycling times and total number of cycles can also be adjusted on a per-assay basis if optimization is needed. Assay performance can vary based on a master mix's chemical formulation. Transitioning a previously optimized qPCR or dPCR assay may warrant changes to the thermal cycling protocol to account for differences in master mix composition. If an assay is initially evaluated using qPCR, then the Absolute Q dPCR master mix could be used during evaluative testing.

Understanding dPCR data

A common visualization of dPCR endpoint data is a plot of the fluorescence signal for each of the thousands of individual microreactions, as shown in Figure 3. This should produce a cluster of higher-fluorescence signals representing microreactions that were positive for the target sequence. This higher-signal cluster should be well-separated from the cluster of lower-fluorescence signals from microreactions not containing the target sequence. The user or the analysis software sets a threshold (cutoff) value that determines whether a microreaction is target-positive (above threshold) or target-negative (below threshold).

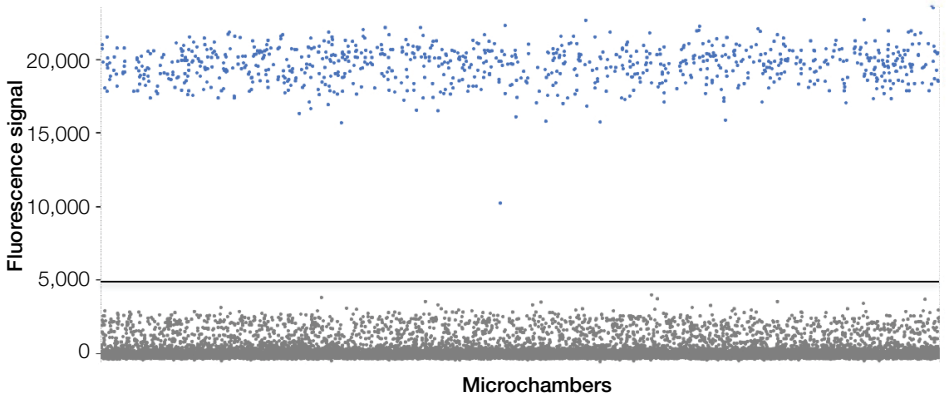

Figure 3. Example of one-dimensional (1D) dPCR scatter plot. Fluorescence signal is plotted for each individual microchamber. The threshold value is indicated by the black horizontal line.

Table 1. Comparison of reaction mix preparation for dPCR and qPCR.

dPCR		qPCR	
Reagent	**Volume per dPCR reaction**	**Reagent**	**Volume per 20 µL reaction**
Absolute Q Universal DNA dPCR Master Mix (5X)	2.0 µL	Applied Biosystems™ TaqMan™ Fast Advanced Master Mix (2X)	10 µL
Primers and probe(s) (20X)	0.5 µL	Primers and probe(s) (20X)	1 µL
Water and sample	7.5 µL	Water and sample	9 µL
Add 9 µL of reaction mix followed by 15 µL of isolation buffer per well of the QuantStudio Absolute Q MAP16 plate.		Add 20 µL of reaction mix to each well of the qPCR plate.	

This conversion of the continuous analog fluorescence signals into binary "yes" or "no" classifications represents the "digital" nature of dPCR. In Figure 3, the threshold is indicated by the horizontal line at 5,000 fluorescence units. The analysis software applies Poisson statistics to convert the counts of positive and negative microreactions to the measured target concentration value.

dPCR results

The measured dPCR quantity is reported as the target concentration in cp/µL. This value reflects the initial target concentration in the dPCR reaction mix that was loaded into the plate. The analysis software has an optional "dilution factor" feature, allowing a user to specify a conversion value to scale the directly measured concentration to the target concentration in the original sample of interest. For example, if 2 µL of nucleic acid sample was combined with 8 µL of additional reagents to create the dPCR reaction mix, this would correspond to a 5-fold dilution of the target. Dilution factors can be defined within the plate setup menu for sample wells, and the software will directly provide the concentration of target in the undiluted sample. If any additional upstream dilutions were performed (for example, to bring the target into the dPCR range), these can also be accounted for by the software for convenient data reporting.

Cluster separation

Data points that fall between the positive and negative clusters are often referred to as "rain" and are commonly encountered in dPCR data analysis (Figure 4). These intermediate values of fluorescence can be attributed to microchambers in which amplification is incomplete or delayed. Rain in dPCR data can be due to a variety of experimental factors, including assay performance issues, differences between target sequences, and secondary structures in the templates. In many cases, rain can be minimized by optimizing the assay design and/or thermal cycling conditions. However, as long as the threshold can accurately differentiate target-positive and target-negative microreactions, the presence of intermediate signal intensities is unlikely to have a significant impact on final quantification.

Some degree of rain may be unavoidable for certain assay designs or sample types. Since rain is often due to incomplete or delayed amplification (i.e., intermediate fluorescence signal representing "weak positives"), setting the threshold beneath these intermediate values may produce more accurate results. Preliminary experiments using control samples such as a dilution series of a positive control can provide empirical evaluation of the adequacy of a proposed threshold value by assessing the observed relative change in target quantification between dilutions. Furthermore, the potential impact of threshold ambiguity in the presence of rain can be evaluated by comparing the concentration results when the threshold is set to a lower versus higher position to assess if this variation is significant relative to the assay's precision needs (Figure 4).

Multiplexing considerations

Developing multiplexed assays is generally simpler for dPCR than for qPCR because minor changes in PCR efficiency do not impact quantification based on endpoint signals. Additionally, dividing the reaction volume into thousands of isolated microreactions reduces the degree of target competition. Although one target could be orders of magnitude more abundant than another within the bulk dPCR reaction mix, they will be close to equal in concentration when colocalized within the same microreaction. Combining multiple targets in the same reaction mix can produce more convenient testing workflows and enable enhanced precision when comparing intra-sample ratios of target concentrations, commonly performed in copy number variation and gene expression analyses.

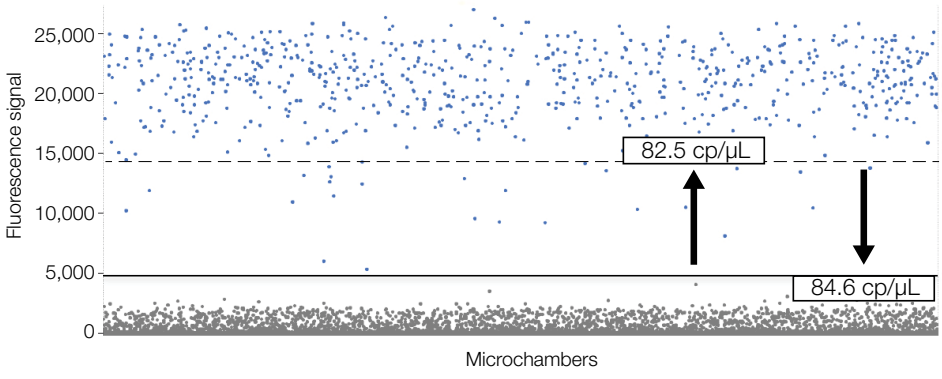

Figure 4. Example 1D dPCR scatter plot with rain. The term "rain" is often used to indicate microreactions with intermediate signal intensity. Rain can reduce confidence in defining a threshold value. The potential impact on target quantification depends on the degree of rain. Two candidate thresholds are shown (solid and dotted horizontal lines) along with the resulting target quantification values based on the threshold position.

Compared to qPCR, dPCR has a narrower dynamic range for target input concentrations. This is especially important when considering a multiplex assay design. If the desired targets are of vastly different concentrations (e.g., more than a 10,000-fold difference), it may not be feasible to quantify both targets within the same reaction. For example, if a higher-abundance target requires further dilution to enter the dPCR range, a lower-abundance target may become over-diluted and no longer detectable.

Conversely, omitting that additional dilution step may produce satisfactory quantification values for the lower-abundance target, but the higher-abundance target might now be above the dynamic range and produce all positive microchambers, precluding accurate quantification. Before defining a multiplex dPCR assay, ensure that the chosen targets have similar enough expected concentrations that they will both fall within the dPCR dynamic range.

Most considerations for qPCR multiplex assay design also apply to dPCR. However, one notable exception is that "primer limitation" should not be employed for dPCR multiplexing. Primer and probe sequences from different assays should be checked for cross-reactivity. Various online resources are available to aid in this step, including our **Multiple Primer Analyzer** tool. This tool also provides estimated melting temperatures of the sequences, a critical consideration when combining different assays to operate within the same thermal cycling conditions.

Assays being considered for multiplexing should ideally produce robust amplification when run in singleplex. A less efficient assay that performs adequately for singleplex dPCR could produce suboptimal results when combined with other assays. Reviewing qPCR amplification curves from a target dilution series run in a singleplex assay can provide helpful insight. Steep amplification curves with high and consistent fluorescence signal plateaus throughout the dilution series are suggestive of robust assays well suited for dPCR multiplexing.

Multiplex dPCR data

For multiplex dPCR assays, two-dimensional (2D) scatter plots present the microchamber fluorescence signal values for a pair of dye labels (Figure 5A). As with 1D plots, each dot represents the fluorescence values from a single microchamber. However, 2D plots enable evaluation of microchambers that are positive for both targets (green cluster), a single target (purple and orange clusters), or neither target (gray cluster). The vertical axis of the 2D plot in Figure 5A corresponds to the JUN dye signal. Figure 5B shows the corresponding 1D scatter plot for the JUN dye, where the upper cluster (red color) represents microchambers positive for the JUN dye–labeled target. The 2D plot separates the JUN dye–positive band of Figure 5B into the single positives (purple cluster) and the VIC and JUN dye double positives (green cluster). Additionally, the 2D view separates the JUN dye–negative band of Figure 5B into the double negatives (gray cluster) and the VIC dye–positives (orange cluster).

Reviewing data within 2D plots can increase accuracy in threshold placement. For some assays, microchambers positive for both targets may be less intense or show an increased degree of rain compared to the signals from chambers positive for only one of the targets. This is often true for competitive assays assessing single-nucleotide variations (Figure 5C). When viewed in the corresponding 1D plot (Figure 5D), the presence of intermediate signals can make it difficult to accurately delineate positive versus negative chambers. However, when viewing the vertical axis of the 2D plot (Figure 5C), it becomes more apparent that the intermediate signal arises from microchambers that are positive for both targets (green cluster). The additional insight provided by the 2D plot can help guide accurate placement of the threshold compared to reviewing 1D plots alone.

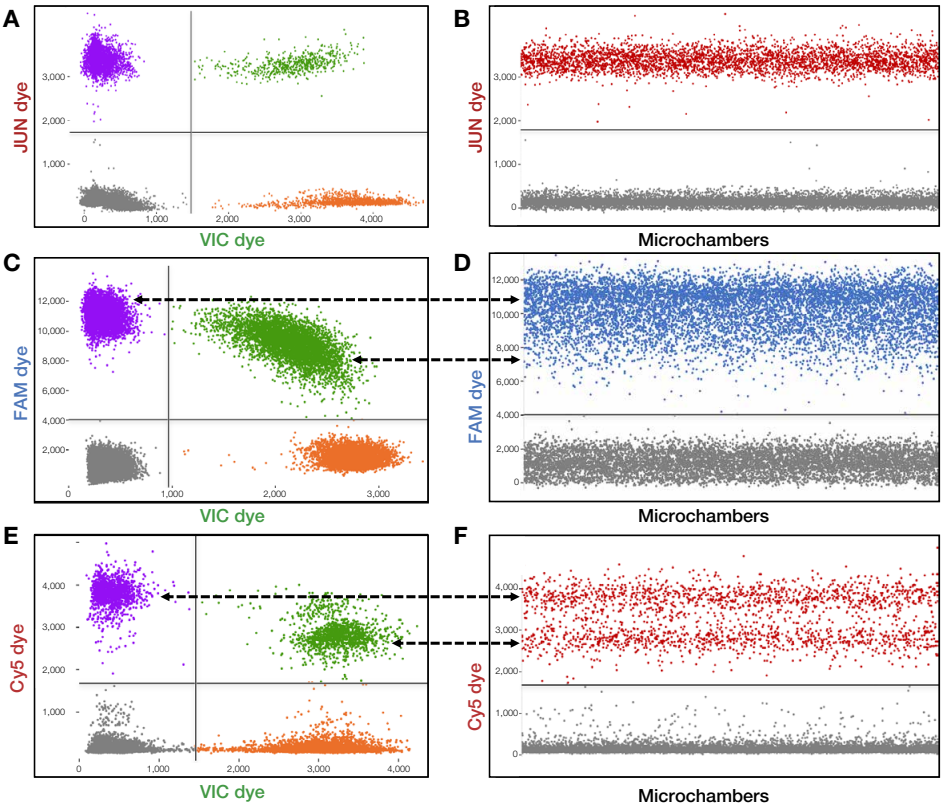

Figure 5. 2D dPCR data plots. (A, C, E) Illustrative 2D data plots for three different assay conditions. **(B, D, F)** For each 2D plot, the corresponding 1D data plot is shown for the dye represented on the vertical axis of the 2D plot. **A** and **B** illustrate good cluster separation and minimal assay competition. **C** and **D** represent a single-nucleotide variation assay (i.e., one shared primer set and two target probes with different dye labels), showing commonly encountered "smearing" of the double-positive cluster for this type of assay design. **E** and **F** illustrate an example of competition between assays when the different targets are colocalized within the same microreaction.

Competition can also occur in a multiplex assay when different independent target sequences colocalize within the same microchamber. This can produce an extra cluster when viewing data in the 1D plot (Figure 5F). From the 1D plot alone, it may not be apparent what the additional middle cluster represents and whether the threshold should be placed above it or below it. The 2D view of the same data (Figure 5E) shows that the middle cluster represents reduced fluorescence signals in the Cy5 dye channel when the chamber also contains the VIC dye–labeled target. Review of the 2D plot clarifies that the Cy5 dye threshold should be below the middle cluster rather than above it.

Conclusion

This guide is intended to help transition existing qPCR and dPCR assays to the QuantStudio Absolute Q Digital PCR System. In general, well-designed assays can be easily transitioned to run on the QuantStudio Absolute Q Digital PCR System with few issues. For more information about dPCR and to view more dPCR resources, including technical notes, application notes, user guides, and webinars, visit **thermofisher.com/dpcr-resources**.

Ordering information

Product	Cat. No.
QuantStudio Absolute Q Digital PCR System	A53267
QuantStudio Absolute Q AutoRun dPCR Suite	A57608
TaqMan Assays, predesigned	See thermofisher.com/taqman
Absolute Q Universal DNA Digital PCR Master Mix (5X)	A72710
QuantStudio Absolute Q MAP16 Plate Kit	A52865
QuantStudio Absolute Q MAP16 Plate Kit and Absolute Q Universal DNA dPCR Master Mix	A40001697
Absolute Q 1-Step RT-dPCR Master Mix (4X)	A55146

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