

## Digital PCR

# Quick reference guide for running existing assays on the QuantStudio Absolute Q Digital PCR System

This reference guide can be used to transition optimized quantitative PCR (qPCR) assays and digital PCR (dPCR) assays to the Applied Biosystems™ QuantStudio™ Absolute Q™ Digital PCR System.

## Key terms

- **Assay:** A combination of forward and reverse primers and a target-specific fluorescent probe.
- **Template:** Purified DNA or cDNA used as input material.
- **Target:** A genomic sequence of interest to be amplified and distinguished with a specific assay.
- **Copy or molecule:** A single, continuous, non-fragmented piece of nucleic acid.
- **Reaction mix:** The final master mix that contains a polymerase, dNTPs, buffer, assay components, and a template. The reaction mix is complete and ready to load into a well on the Applied Biosystems™ QuantStudio™ Absolute Q™ MAP16 Plate.
- **QuantStudio Absolute Q MAP16 Plate:** A dPCR plate with 16 sample arrays, each comprised of 20,480 fixed microchambers.
- **Well:** Port upstream of one QuantStudio Absolute Q MAP16 dPCR array.

## Key considerations before getting started

As a general principle, well-designed Applied Biosystems™ TaqMan™ qPCR assays and existing dPCR assays can be transitioned to the QuantStudio Absolute Q Digital PCR System with few issues. This is because dPCR measurements taken at the endpoint depend less on high reaction efficiency than qPCR measurements. Using multiple assays in a single dPCR reaction, known as multiplexing, can increase the number of targets detected per reaction. Multiplexing different amplicons in a single

dPCR reaction is often as simple as mixing assays with different fluorophores without primer limitation. However, every assay should be tested individually in a simplex format with appropriate positive controls. This will ensure concordance between the results of both simplex and multiplex dPCR reactions.

## Assay

For a final reaction mixture, we recommend a concentration of 900 nM each for the forward and reverse primers and a probe concentration of 250 nM. These concentrations are recommended for both dPCR and qPCR. If the primer concentrations in an existing qPCR assay are lower, the primer concentrations should be increased to 900 nM for dPCR. The concentration of a probe labeled with Applied Biosystems™ FAM™ dye in the final reaction mix should always be limited to a maximum of 250 nM to reduce the risk of saturation, which can result in a suboptimal signal.

## Template

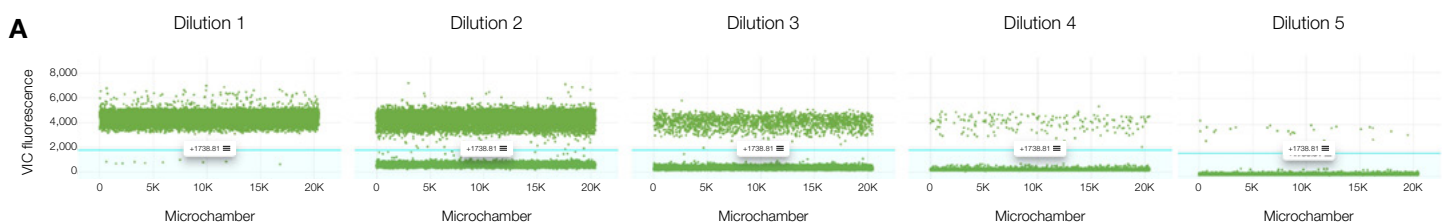
A bulk dPCR reaction mix is digitized across many thousands of microchambers, which divides the target template into individual reactions. In an ideal dPCR reaction, each microchamber contains one, several, or zero target molecules. After thermal cycling to the endpoint, each reaction is read to determine whether the microchamber is positive or negative for the target. The presence of both positive and negative microchambers is crucial for final quantification of the target. When testing an assay using dPCR, it is thus important to establish the approximate number of target copies per genome or sample of interest. For example, there are approximately 300 haploid molecules or copies of a given target in 1 ng of human genomic DNA, and there is one target of interest in a single synthetic plasmid.

## How much template should I use as input material for my first reaction?

Poisson statistics are used in dPCR to enable absolute quantification of specific targets. This simple statistical calculation enables robust quantification of the total number of targets that are present in a reaction without requiring standard curves or reference samples. For this measurement to be accurate, the reaction must have both target-positive and target-negative microchambers. When testing or optimizing a reaction for the first time, an ideal starting range for the target is 200 to 20,000 copies (cp) per 9  $\mu$ L of reaction mix. Using an input concentration within this range will ensure both target-positive and target-negative microchambers are present in the reaction. The ideal input quantity may be above or below this range depending on your experimental needs and the properties of your sample. For instance, a sample should not be diluted when the target of interest is rare or low in abundance. Online calculators like ours at [thermofisher.com/dna-calculator](https://thermofisher.com/dna-calculator) can be used to help determine an appropriate template input quantity.

## How does target concentration impact dPCR data?

As the concentration or number of target copies in a reaction decreases, the number of positive microchambers in the reaction will also decrease. The software will report the concentration of the target in the reaction mix in cp/ $\mu$ L. To demonstrate the data output from a dPCR reaction with varying input concentrations, a 5-point 10-fold dilution series was prepared using an adeno-associated virus (AAV) control. One-dimensional dPCR data plots generated with the Applied Biosystems™ QuantStudio™ Absolute Q™ Digital PCR System software are shown in Figure 1A. The concentration and number of AAV target copies per 9  $\mu$ L of reaction mix reported by the software are listed in Figure 1B. Dilution 1 was the highest concentration point and contained over 200,000 copies of the target per dPCR reaction. Nearly all microchambers were target-positive at this concentration with high fluorescence values. As the target concentration decreased, the number of highly fluorescent target-positive microchambers decreased. In this example, target concentrations in dilution 2 through dilution 4 would be considered ideal for initial assay testing.



## B

AAV target	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5
Target concentration	20,133 cp/ $\mu$ L	2,193 cp/ $\mu$ L	217 cp/ $\mu$ L	21 cp/ $\mu$ L	2 cp/ $\mu$ L
Total number of target copies per 9 $\mu$ L	201,330	21,930	2,170	210	20

**Figure 1. One-dimensional fluorescence dot plots for dPCR quantification of an AAV target in a 10-fold dilution series. (A)** Absolute quantification of AAV copy number was possible over a dynamic range spanning four orders of magnitude using the QuantStudio Absolute Q Digital PCR System. **(B)** Concentration and total copies per reaction of the AAV target across the 10-fold dilution series.

**Table 1. Differences between preparation of dPCR and qPCR reaction mixes.**

dPCR		qPCR	
Prepare PCR reaction mix by adding water, master mix, primers, probe(s), and DNA or cDNA.			
Reagent	Volume ( $\mu$ L) per reaction (1X)*	Reagent	Volume ( $\mu$ L) per 20 $\mu$ L reaction
Applied Biosystems™ Absolute Q™ DNA Digital PCR Master Mix (5X)	2	Applied Biosystems™ TaqMan™ Fast Advanced Master Mix (2X)	10
Primers and probe(s) (20X)**	0.5	Primers and probe(s) (20X)*	1
Water and sample	7.5	Water and sample	9
Add 9 $\mu$ L of reaction mix followed by 15 $\mu$ L of isolation buffer to a well in the MAP16 plate.		Add 20 $\mu$ L of reaction mix to each well in a standard 96-well qPCR plate.	

\* After calculating the number of reactions required, prepare the dPCR mix and scale up each component by 10% for overage. Prepare enough mix for multiple reactions to avoid pipetting volumes of less than 1  $\mu$ L.

\*\* If you are running a custom assay, use the recommended concentrations of Applied Biosystems™ TaqMan™ primers (900 nM) and probes (250 nM).

## Preparing dPCR reaction mixes

### Template preparation

In the master mix quick reference guide, users are instructed to assemble the complete dPCR reaction mix and centrifuge it. Physical debris can interfere with the distribution of the reaction mix into the microchambers during digitization. Templates may be centrifuged before they are added to the reaction mix to help reduce the risk of carrying physical debris over to the reaction mix. The final reaction mix must be centrifuged to remove particulates and other debris before it is added to the sample wells in the QuantStudio Absolute Q MAP16 dPCR plate.

### Digestion or fragmentation

Restriction digestion or fragmentation of the input template may be necessary if you are running a copy number variation (CNV) assay. CNV assays that target linked tandem sequences require digestion to ensure accurate analysis of the individual target genetic region and a true copy number count. If using enzymatic restriction digestion, the restriction enzyme you select should have cut sites surrounding your target sequence but outside the target amplicon region.

### Reaction mix preparation

Preparing a reaction mix for dPCR on the QuantStudio Absolute Q dPCR system is similar to preparing a qPCR reaction mix (Table 1). The starting concentrations of the primers and probe(s) may be adjusted as needed during the optimization process.

**Note: Careful selection of properly calibrated pipettes is key for dPCR success. Use high-quality plasticware that is free of nucleases, as they can impact template quality.**

Carefully mixing your samples before loading them onto the QuantStudio Absolute Q MAP16 dPCR plate is crucial to ensure all reaction components are distributed evenly in all of the microchambers.

### Thermal cycling conditions

The thermal cycling set points for your assay(s) should remain the same or similar across platforms. However, the times for denaturation and annealing/extension may be reduced due to the smaller dPCR reaction volume and physical contact between the microchambers and the thermal surface. We recommend starting with the default cycle times of 5 seconds for denaturation and 15 seconds for annealing (Table 2).

**Table 2. Default thermal cycling protocol for dPCR on the QuantStudio Absolute Q dPCR system.**

Step	Temperature	Time	Cycles
Preheating	96°C	10 min	Hold
Denaturing	96°C	5 sec	40
Annealing and extension	60°C	15 sec	

**Note: It is critical to select the correct dye(s) for your assay to ensure you collect high-quality data, and it is not possible to select a different dye after the run.**

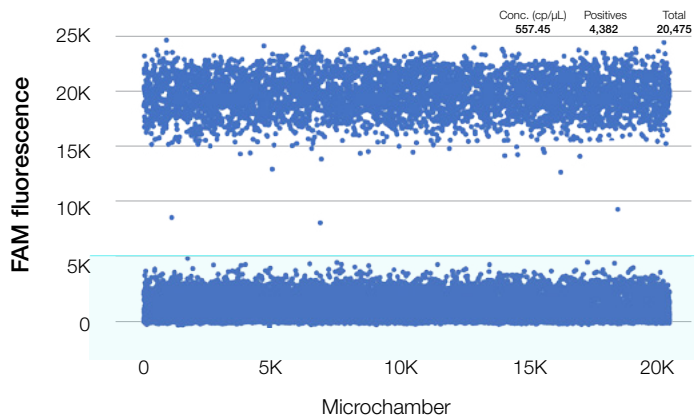
The thermal cycling parameters for annealing and extension may need to be adjusted for existing or custom assays depending on factors like melting temperature. Denaturation, annealing, and extension times may be increased for longer amplicons (>200 bp) if the data indicate adjustments are necessary. Amplicons as long as 200 bp have been used successfully with the extension times listed in Table 2.

### Understanding digital PCR data

Identification of positive and negative dPCR microchambers is based on a fluorescence threshold set by the user or analysis software. The fraction of positive microchambers is then fitted to a Poisson distribution to determine the absolute target copy number (cp/μL) in the input reaction mix.

### Simplex dPCR data

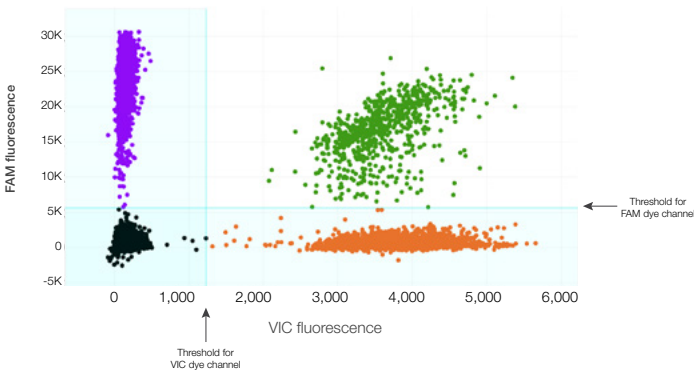
A well-performing dPCR assay will make it easy to distinguish between microchambers that are positive for the target and those that are negative. This in turn will make setting the fluorescence threshold fairly simple. Figure 2 is a dPCR dot plot showing clear separation between positive and negative microchambers across a fluorescence threshold of 5,000.



**Figure 2. Example of a dPCR dot plot showing good separation of positive and negative microchambers.** Each microchamber accepted for analysis is plotted along the x-axis, and the fluorescence intensity from each microchamber is plotted along the y-axis. The fluorescence threshold is indicated by the light blue line at 5K FAM fluorescence. Microchambers with fluorescence intensities above the threshold are counted as positive for the target, while those with fluorescence intensities below the threshold are counted as negative.

## Multiplex dPCR data

Setting the threshold for a multiplex dPCR assay, or an assay with more than one fluorescently labeled probe, should be performed by visualizing the data in a two-dimensional (2D) plot. When thresholding for two targets in 2D, four clusters can be identified in the plot. A 2D plot of multiplex data with thresholds of 5,000 for FAM dye and 1,000 for Applied Biosystems™ VIC™ dye is shown in Figure 3. The black dots represent microchambers that are negative for both targets. The purple dots represent microchambers that are positive only for the target labeled with FAM dye, while the orange dots represent microchambers that are positive only for the target labeled with VIC dye. The green dots represent microchambers that are positive for both targets, which indicates both targets are present.



**Figure 3.** dPCR plot of data from a multiplex dPCR assay with two targets and two probes labeled with FAM dye (y-axis) and VIC dye (x-axis). The fluorescence intensities of both dyes are plotted for each analyzed microchamber. The purple dots above the horizontal threshold represent microchambers that are counted as positive for the probe labeled with FAM dye. The orange dots beyond the vertical threshold represent microchambers that are counted as positive for the probe labeled with VIC dye. The green dots represent microchambers that are counted positive for both probes, which indicates both targets are present.

## Double or multi-positive signals

When performing multiplex dPCR, it is typical to detect microchambers that contain more than one target. As the target concentrations increase, the number of microchambers that test positive for multiple targets will also increase. In Figure 3, microchambers that are positive for both targets are represented by the green dots, and the quantity of double-positive microchambers can be easily counted with the software. Thresholding for multiplex assays in 2D will help you best identify an appropriate place for the fluorescence threshold for each channel.

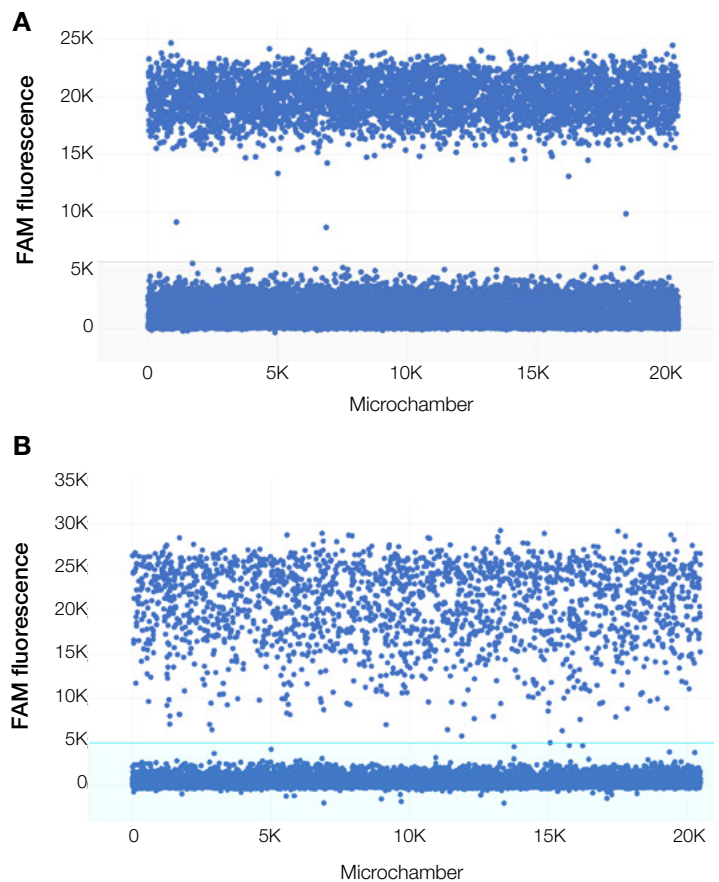
## Rain

Data points that fall between the negative and positive clusters in a 2D dPCR fluorescence plot are often referred to as rain, which is a common phenomenon (Figure 4). Rain at lower fluorescence intensities 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 transcripts, and secondary structures in the templates. Rain can be minimized in some cases by optimizing the assay. However, as long as the threshold can be placed between the clusters of higher fluorescence and lower fluorescence, counting the microchambers as positive or negative does not have a significant impact on final quantification. When rain is due to secondary structures, which is common with plasmid controls, it can be reduced or eliminated by digesting the templates with restriction enzymes or briefly sonicating the samples to increase target accessibility.

## Quality control checks

The QuantStudio Absolute Q MAP16 Plate has an excellent microchamber fill rate. The total number of microchambers accepted for analysis should be above 19,000. To check this, note the numbers listed in the Total column shown in Figure 5. If fewer than 19,000 microchambers are full, two methods can be used to evaluate the quality of the data to determine if it is high enough for downstream analysis.



**Figure 4.** Example 1D dPCR plots with varying degrees of “rain” resulting from incomplete or delayed amplification. (A) dPCR data showing minimal rain. (B) dPCR data showing a moderate amount of rain.

## Quality control (QC) plots

The passive reference dye in Applied Biosystems™ Absolute Q™ dPCR master mixes is used by the analysis software to identify microchambers that have been properly filled. Select the QC button on the top left side of the interface to view the fill for each sample across the microchamber array and identify microchambers that have been accepted or rejected based on the signal from ROX passive reference dye. If the software reports “NaN”, it means that many of the microchambers have not been categorized successfully and the sample must be re-run. Fluorescence plots reflecting even and uneven distribution of reference dye are shown in Figure 6.

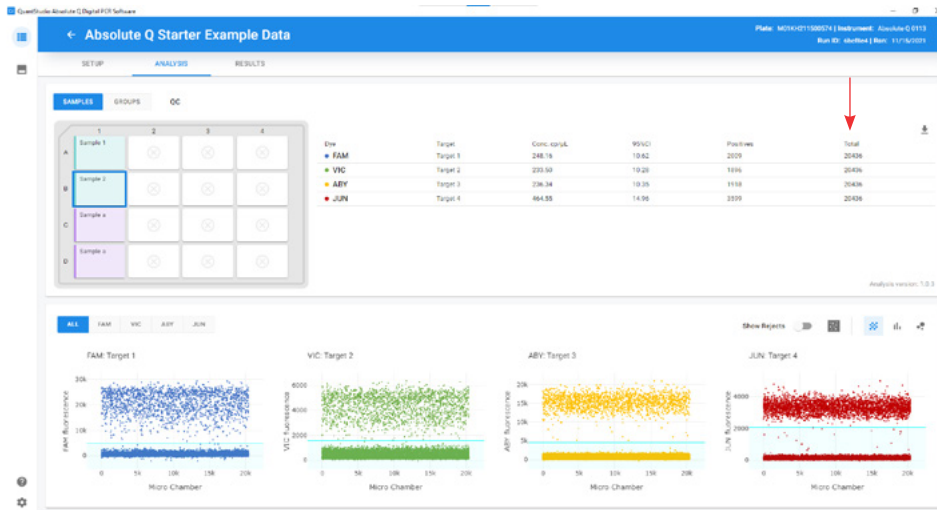


Figure 5. Examples of data obtained with the QuantStudio Absolute Q Digital PCR System. The total number of microchambers that contain a specific fluorescent probe can be found in the Total column (under the red arrow).

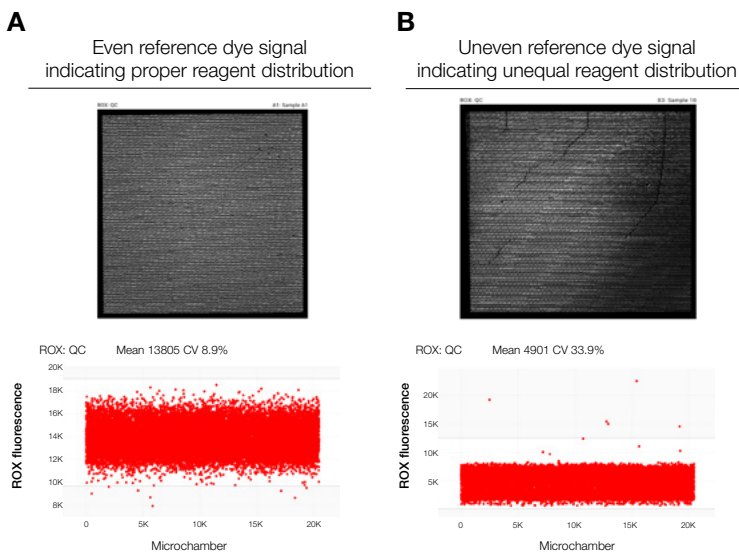


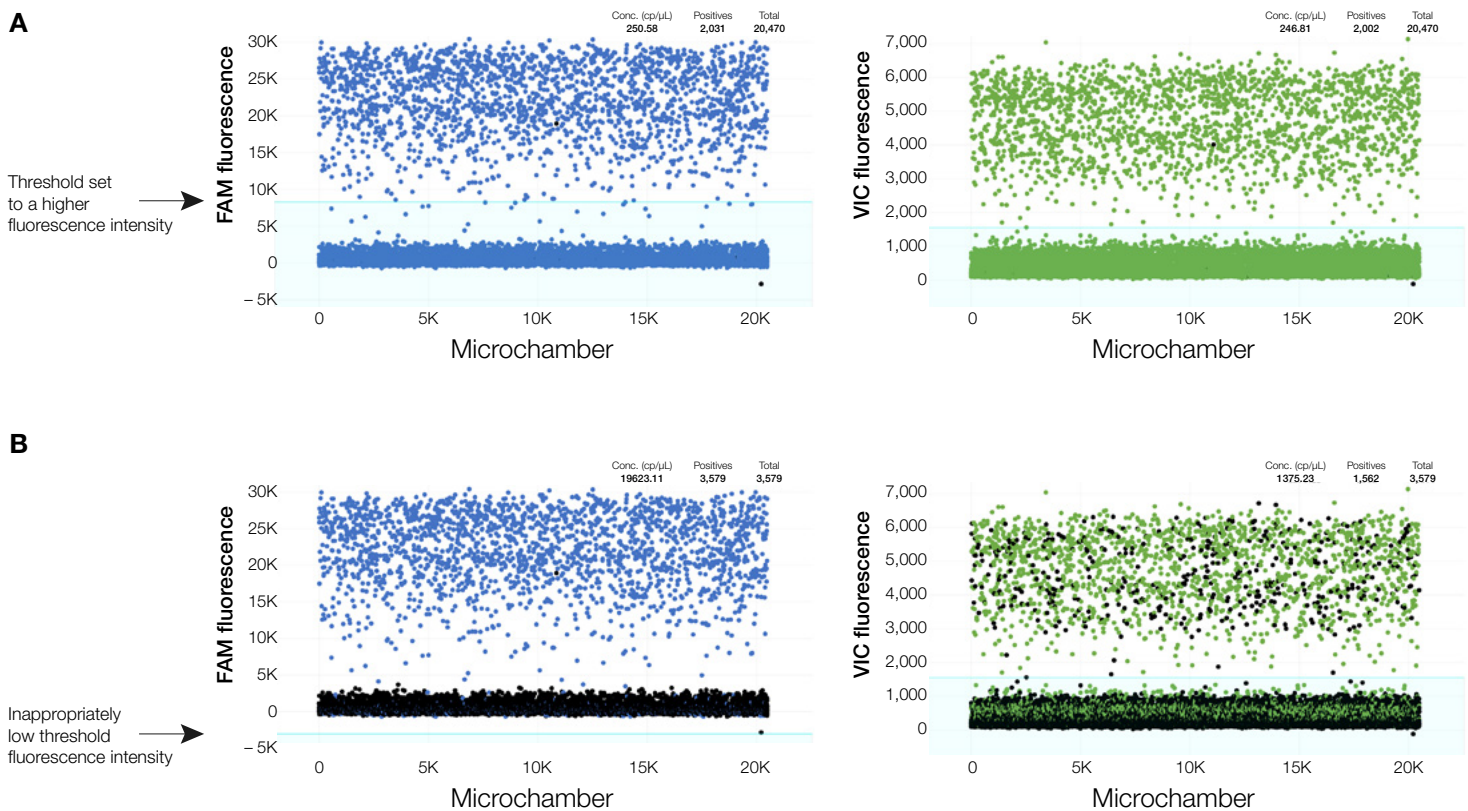
Figure 6. Distribution of passive reference dye in the QuantStudio Absolute Q MAP16 Plate. (A) Even distribution of reagents in the plate is indicated by fluorescence intensities within the expected range and a CV of less than 10%. (B) Lower than expected fluorescence and a CV of 33.9% indicate unequal distribution of reagents in the plate.



## Improper thresholding

Microchambers with fluorescence intensities above the positive threshold are evaluated with an artificial intelligence (AI)-powered algorithm that identifies and rejects any microchamber that does not satisfy the criteria for a true positive. For instance, an image artifact that may appear to be a fluorescence signal will be rejected and will not generate a false positive. This means that improper thresholding can cause otherwise acceptable microchambers to be rejected. The data shown in Figure 7 are for the same sample from a multiplex assay run with FAM and VIC dyes. The positive threshold for the FAM channel is set above 5,000 in Figure 7A. It is set to below zero in Figure 7B, which

places many negative microchambers above the threshold. The classification algorithm inspects all microchambers that are above the threshold and determines that the low-fluorescence “negative” microchambers do not fit the criteria for true positives. Lowering the threshold results in rejection of the negative microchambers by the classifier across both channels for FAM and VIC dyes, because they are positioned above the positive threshold but do not look like true positive microchambers. The rejected data points are shown in black (Figure 7B). To resolve rejection of negative microchambers, the FAM channel threshold should be set between the positive and all of negative microchamber clusters.



**Figure 7. Fluorescence intensity dot plots with appropriate and inappropriate thresholds. (A)** A properly thresholded multiplex dPCR reaction with FAM and VIC dye thresholds set between the high-fluorescence and lowest-fluorescence clusters. **(B)** The threshold for FAM fluorescence is set too low, and negative microchambers are erroneously counted as positive. The software algorithm identifies and rejects microchambers that do not satisfy the criteria for being true positives. The rejected data points are shown in black.

## Summary

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

 Learn more at [thermofisher.com/absoluteq](https://thermofisher.com/absoluteq)

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