Vericheck ddPCR™ Replication Competent AAV Kit User Guide

Catalog #	Description
12019571	**Vericheck ddPCR Replication Competent AAV Kit**, 100 x 20 µl reactions, includes assay, supermix, positive control, negative control, and internal control

For research purposes only.

Product Description

Replication competent viruses (RCVs) are virus particles that can infect cells and replicate to produce additional infectious viral particles. These may be generated after the introduction of an adeno-associated virus (AAV) vector into the target cells by homologous or nonhomologous recombination. Samples are collected during different stages of the viral production workflow to test for replication competence.

The Vericheck ddPCR Replication Competent AAV (RCAAV) Kit can be used to confirm the presence or absence of replication competent AAV during the production of cell and gene therapy drug products. The Vericheck ddPCR RCAAV Kit is compatible with AAV serotypes 1–10.

Performance Characteristics

Analytical performance characteristics are summarized in Table 1. The dynamic range, limit of detection (LOD), and limit of quantitation (LOQ) were determined using a DNA plasmid sample or a gBlock sample serially diluted in TE buffer with carrier. The kit is linear throughout the dynamic range. LOD is set as the lowest concentration sample detected in ≥95% of tests. LOQ is set as the lowest concentration sample detected in ≥99% of tests. The kit was tested with nuclease-free water (negative control) and no false positives were observed at the 95th percentile of tests, supporting a limit of blank (LOB) of zero copies/µl. Specificity was tested using genomic DNA digested with AluI restriction enzyme. The maximum allowable concentrations of common inhibitors in the starting sample prior to extraction are listed in Table 2.

Table 1. Analytical performance summary.

Dynamic range	0.50 to 7,500 copies/µl*
Linearity	Slope = 0.99, R^2 = 1.00
LOD	0.50 copies/µl*
LOQ	0.60 copies/µl*
LOB	0.00 copies/µl*
Specificity	No detection of HEK293, CHO, human, *E. coli*, or *Mycoplasma* genomic DNA

* Copies/µl refers to copies/µl in the 20 µl ddPCR reaction.

Table 2. Inhibitor tolerance: Maximum allowable concentrations of common inhibitors in the final ddPCR reaction well.

Inhibitor	Allowable Concentration in Well
97.5% PBS + 2.5% HSA	7.75%
Pluronic F-68	1.25%
PEI	1 pg/µl
DMSO	4%

Kit Contents

The kit contents are provided in Table 3. Keep at –20°C for long-term storage or at 4°C for up to 2 weeks. The kit provides sufficient reagents for 96 reactions, either 1 plate with up to 30 samples + controls or 2 plates with up to 14 samples per plate + controls.

Table 3. Kit contents.

Components	Tubes/Kit	Volume/Tube
ddPCR Supermix for Residual DNA Quantification (2x)	2	0.75 ml
ddPCR RCAAV Internal Control (20,000 copies/µl)	1	0.15 ml
ddPCR RCAAV Positive Control	1	0.08 ml
Nuclease-Free Water (Negative Control)	1	1.50 ml
ddPCR RCAAV Assay (20x)	1	0.15 ml

Table 4. Additional materials required.*

System	Instruments	Consumables and Reagents
🟧 QX200™ Droplet Digital™ PCR System	• QX200 Droplet Generator (catalog #1864002) • QX200 Droplet Reader (#1864003) • C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module (#1851197) • PX1 PCR Plate Sealer (#1814000)	• Droplet Generation Oil for Probes (#1863005) • ddPCR Droplet Reader Oil (#1863004) • ddPCR 96-Well Plates (#12001925) • DG8 Cartridges (#1864008) • DG8 Gaskets (#1863009) • DG8 Cartridge Holder (#1863051) • ddPCR Buffer Control for Probes (#1863052) • PCR Plate Heat Seal (#1814040)
🟩 QX200 AutoDG™ Droplet Digital PCR System	• Automated Droplet Generator (#1864101) • QX200 Droplet Reader (#1864003) • C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module (#1851197) • PX1 PCR Plate Sealer (#1814000)	• Automated Droplet Generation Oil for Probes (#1864110) • ddPCR Droplet Reader Oil (#1863004) • ddPCR 96-Well Plates (#12001925) • DG32 Automated Droplet Generator Cartridges (#1864108, 1864109) • ddPCR Buffer Control for Probes (#1863052) • Pipet Tips for AutoDG System (#1864120, 1864121) • Pipet Tip Waste Bins for the AutoDG System (#1864125) • PCR Plate Heat Seal (#1814040)
🟦 QX ONE™ Droplet Digital PCR System	• QX ONE Droplet Digital PCR System (#12006536) • PX1 PCR Plate Sealer (#1814000)	• QX ONE Droplet Generation Oil for Probes (#12006058) • QX ONE Droplet Reader Oil (#12006057) • GCR96 Cartridges (#12006858, 12006859) • ddPCR Buffer Control for Probes (#1863052) • GCR96 Foil Seal (#12006843)
🟥 QX600™ AutoDG Droplet Digital PCR System	• Automated Droplet Generator (#1864101) • QX600 Droplet Reader (#12013328) • C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module (#1851197) • PX1 PCR Plate Sealer (#1814000)	• Automated Droplet Generation Oil for Probes (#1864110) • ddPCR Droplet Reader Oil (#1863004) • ddPCR 96-Well Plates (#12001925) • DG32 Automated Droplet Generator Cartridges (#1864108, 1864109) • ddPCR Buffer Control for Probes (#1863052) • Pipet Tips for AutoDG System (#1864120, 1864121) • Pipet Tip Waste Bins for the AutoDG System (#1864125) • PCR Plate Heat Seal (#1814040)
Sample preparation	• C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module (#1851197)	• Poly(A), polyadenylic acid (MilliporeSigma, #10108626001) • DNA Suspension Buffer, DNase/RNase Tested, PCR Grade (10 mM Tris, pH 8.0, 0.1 mM EDTA) (Alpha Teknova, Inc., #T0223) • Poloxamer 188 (Pluronic F-68) Non-Ionic Surfactant (ThermoFisher Scientific Inc., #24040032) • DNase I (RNase-free) (10x DNase I Reaction Buffer is included) (New England Biolabs Inc., M0303)

* Adjustable pipets (Rainin or Eppendorf) can be used with the consumables and reagents listed.

Required Equipment, Reagents, and Consumables
See Table 4 for required materials that are not included in the kit.

Sample Preparation
The dynamic range of the kit is 0.5–7,500 copies/µl. For an unknown sample, it is suggested to prepare and test up to six 10-fold serial dilutions to ensure that the sample falls within the dynamic range. Table 2 contains the maximum allowable concentration of common inhibitors in the final ddPCR reaction well.

AAV samples should be treated with DNase I and heat lysed prior to ddPCR testing. The recommended workflow for AAV sample preparation is summarized in Figure 1. First, prepare the reagents for sample preparation, as described on page 3. Then, proceed to sample dilution, DNase treatment, and capsid heat lysis.
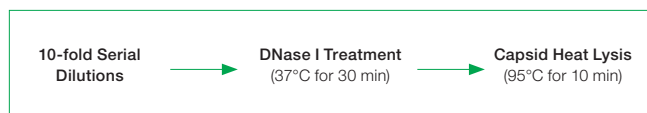

Fig. 1. Sample preparation overview for RCAAV samples.

Stock Reagent Preparation

Stock reagents can be prepared in bulk ahead of time and stored at 4°C. Follow these steps to prepare a poly(A) stock solution and poly(A)+ buffer solution for use with AAV sample preparation.

Poly(A) Stock Solution

1. Resuspend 100 mg poly(A) to a concentration of 50 mg/ml by adding 2 ml of DNA Suspension Buffer to the vial of poly(A).

2. Thoroughly vortex the tube to ensure the poly(A) is resuspended.

3. Store the poly(A) stock solution at 4°C.

Poly(A)+ Buffer Solution

Poly(A)+ buffer solution is 100 ng/µl poly(A) and 0.01% Pluronic F-68 in DNA Suspension Buffer. The volumes below are provided as an example and can be scaled up or down as necessary.

1. To 250 ml DNA Suspension Buffer, add 0.5 ml of the 50 mg/ml poly(A) stock solution and 0.25 ml of 10% Pluronic F-68.

2. Mix well to combine.

3. Store poly(A)+ buffer solution at 4°C.

Fresh Reagent Preparation
1% Pluronic F-68 Solution

Important: Prepare a fresh 1% Pluronic F-68 solution for DNase treatment prior to each round of sample preparation.

1. Add 10 µl of 10% Pluronic F-68 to 90 µl nuclease-free water. The volumes can be scaled up to account for running more DNase reactions.

2. Mix well to combine.

Sample Preparation

1. Make 1:10 serial dilutions of the sample using poly(A)+ buffer. For an unknown sample, it is suggested to prepare and test up to six 10-fold serial dilutions.

2. For each dilution of the original sample to be tested, digest unencapsidated DNA using DNase I. Each dilution to be tested is one DNase reaction.

   a. Prepare a master mix for DNase digestion according to Table 5.

      i. Add the components in the order listed in the table.

      ii. Pulse vortex and briefly microcentrifuge.

Table 5. Preparation of the master mix for DNase reaction.

Component	Volume per 1 reaction, µl*
Water	23
10x DNase I Reaction Buffer	4
1% Pluronic F-68 solution	4
DNase I (2 U/µl)	5
Total volume	**36**

* Multiply the volumes by the number of DNase reactions plus 1 extra reaction for overage.

   b. Aliquot 36 µl of the DNase master mix into individual tubes for DNase treatment.

   c. Add 4 µl of AAV sample to the individual tubes. Flick several times to mix, then microcentrifuge to bring the solution to the bottom of the tubes.

   d. Incubate samples in a C1000 Touch Thermal Cycler at 37°C for 30 min, then cool to 4°C at 3°C/sec ramp rate. Use a heated lid set to 40°C and set the sample volume to 40 µl.

   e. DNase treated samples can be stored for future use at −20°C for short-term or at −80°C for long-term storage.

3. Lyse AAV capsids just prior to setting up the ddPCR reactions.

   a. Pulse spin tubes with the DNase-treated sample in a microcentrifuge to ensure that the solution is at the bottom of the tubes.

   b. Incubate samples in C1000 Touch Thermal Cycler at 95°C for 10 min, then cool to 4°C at a 3°C/sec ramp rate. Use a heated lid set to 105°C and set the sample volume to 40 µl.

   c. Thoroughly mix the samples by flicking the tubes after removing them from the PCR instrument. Microcentrifuge to bring the liquid to the bottom of the tubes.

   d. Proceed to reaction setup.

Plate Setup

An example plate setup is shown in Figure 2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Pos Ctrl	Pos Ctrl	Pos Ctrl									
B	Sample 1	Sample 1	Sample 1									
C	Sample 2	Sample 2	Sample 2									
D	Sample 3	Sample 3	Sample 3									
E	Sample 4	Sample 4	Sample 4									
F	Sample 5	Sample 5	Sample 5									
G	Sample 6	Sample 6	Sample 6									
H	Neg Ctrl	Neg Ctrl	Neg Ctrl									

Fig. 2. Example plate configuration for controls and 6 samples. Columns 4–12 can be used to test additional samples. Three replicate wells per sample and control are recommended.

Tip: Any unused well in a column from which droplets will be generated must be filled with 1x ddPCR Buffer Control for Probes prior to droplet generation.

- **Sample:** Lysed AAV sample is added to the ddPCR reaction. The RCAAV signal is read in channel 1 (FAM). Internal control is added to the master mix and is read in channel 2 (HEX).

- **Pos Ctrl (ddPCR RCAAV Positive Control):** Instead of sample, positive control is added to the ddPCR reaction. The positive control signal is read in channel 1 (FAM). Internal control is added to the master mix and is read in channel 2 (HEX).

- **Neg Ctrl (Nuclease-Free Water):** Instead of sample, nuclease-free water is added to the ddPCR reaction. Internal control is added to the master mix and is read in channel 2 (HEX).

Reaction Setup

Table 6. Preparation of the master mix containing supermix, RCAAV assay, and internal control.

Components	Volume for 1x Reaction, µl	Volume for 48x Reactions + Overage, µl*
ddPCR Supermix for Residual DNA Quantification	10	680
ddPCR RCAAV Assay	1	68
ddPCR RCAAV Internal Control	1	68
Total volume	12	816

* 14 samples + 1 positive control + 1 negative control = 16 total × 3 replicates each = 48 total reactions. Here, the master mix is prepared with 48 µl overage. It is recommended to prepare the master mix with ~40–50 µl overage.

Table 7. Preparation of the reaction mixes.

Components	Volume for 1x Reaction, µl	Volume for 3x Reactions + Overage, µl*
Master mix (from Table 5)	12	48
Sample or ddPCR RCAAV Positive Control or Nuclease-Free Water (Negative Control)	8	32
Total volume	20	80

* 3 replicate wells are prepared together. The reaction mix is prepared with 1 additional reaction as overage.

Procedure

1. Determine the plate layout prior to setting up the reaction mix. Each sample and control should have three replicate wells. An example plate configuration is shown in Figure 2.

2. Thaw all kit components to room temperature. Mix all tubes thoroughly by vortexing at maximum speed for 15 sec to ensure homogeneity. Centrifuge briefly to collect the contents at the bottom of each tube.

   Tip: A thorough vortex (maximum speed for 15 sec) of each component at this step is essential.

3. Ensure samples are thawed to room temperature and thoroughly mixed.

4. Assemble a master mix containing ddPCR Supermix for Residual DNA Quantification, ddPCR RCAAV Assay, and ddPCR RCAAV Internal Control according to the master mix setup guidelines in Table 6. Vortex the master mix thoroughly at maximum speed for 15 sec.

5. Prepare reaction mix for each set of three replicate wells by combining master mix (from step 4) with the sample, controls, or water.

   a. Use the volumes in Table 7 to prepare the sample, negative control, and positive control reaction mixes.

   b. Mix each tube thoroughly by vortexing at maximum speed for 15 sec, and centrifuge briefly to collect the contents at the bottom of each tube.

   Tip: If several reaction mixes are being prepared at once, they can be made in a column of a 96-Well ddPCR Plate, mixed thoroughly, and the reaction mix from each well of the column can be transferred simultaneously to the neighboring columns using a multichannel pipettor.

6. Droplet generation steps differ slightly depending on the system in use. Transfer the reaction mix from the tubes or plate prepared in step 5 to the appropriate Droplet Generation Cartridge or ddPCR Plate and generate droplets as follows for the system in use:

   Tip: Once droplets have been generated, do **not** centrifuge the final 96-well plate containing the droplets.

For manual droplet generation
(▪ QX200 ddPCR System):

a. Load 20 µl of each reaction mix into the sample wells of a DG8 Cartridge preloaded in a DG8 Cartridge Holder. The orientation of the Manual DG8 Cartridge should mirror the way the droplets will be transferred to the final 96-well plate.

b. Add 20 µl of 1x buffer control to any unused wells from which droplets will be generated.

c. Load 70 µl of Droplet Generation Oil for Probes into the oil wells.

d. Hook the gasket over the Cartridge Holder and place into the instrument. For detailed instructions, refer to the QX200 Droplet Generator Instruction Manual (10031907).

For automated droplet generation (▪ QX200 AutoDG ddPCR System or ▪ QX600 AutoDG ddPCR System):

a. Load 22 µl of each reaction mix into the wells of a 96-Well ddPCR Plate.

b. Add 22 µl of 1x buffer control to any unused wells in a column from which droplets will be generated. If an entire column is unused, no buffer control is required in those wells.

c. Seal the plate using the PX1 PCR Plate Sealer at 180°C for **5 sec**, allow to cool briefly, vortex at maximum speed for 15 sec, and centrifuge at 1,000 rcf for 1 min. Visually verify that all the liquid is at the bottom of the well.

d. Place the sealed plate in the Automated Droplet Generator and follow instructions in the Automated Droplet Generator Instruction Manual (D112916).

For the ▪ QX ONE ddPCR System:

a. Load 20 µl of each reaction mix into the wells of a GCR96 Cartridge.

b. Droplet generation occurs in sets of two columns (for example, droplets for columns 1 and 2 are generated simultaneously). Add 20 µl of 1x buffer control to any unused wells in each set of columns from which droplets will be generated.

c. Seal the plate using the PX1 PCR Plate Sealer at 180°C for **0.5 sec**, flip plate 180° and repeat sealing, allow to cool briefly, and centrifuge at 1,150 rcf for 30 sec.

d. Follow subsequent instructions for loading the plate as specified in the QX ONE Droplet Digital PCR System and QX ONE Software User Guide (10000116512). The Vericheck ddPCR RCAAV Kit is compatible with QX ONE Software v1.2 or higher (all editions). Use the appropriate thermal cycling conditions as specified in Table 8.

Important: When setting up the plate template, first select the wells to include/exclude under the Exclude tab. Then move to the Edit tab and designate the following:

▪ Experiment Type: Direct Quantification (DQ)

▪ Sample Descriptions: Determined by user

▪ Sample Type: Determined by user (positive control wells should be designated as Pos Ctrl)

▪ Supermix: ddPCR Supermix for Residual DNA Quantification

▪ Assay Type: Single Target per Channel

▪ Target Name(s): Determined by user

▪ Target Type: Unkn

▪ Signal Ch1: FAM

▪ Signal Ch2: HEX

Press Apply and then Save the template. On the Run Setup screen, name the plate and select the supermix. Press Start Run. See Figure 3 for an example.

Thermal Cycling

Follow the instructions for thermal cycling based on the system in use:

▪ **For the ▪ QX200 ddPCR System**, after manual droplet generation with the QX200 Droplet Generator, carefully transfer each column of the droplet emulsions into a clean ddPCR 96-Well Plate using a P50 multichannel pipettor. Each well of droplet emulsions is approximately 40 µl. Seal the plate using the PX1 PCR Plate Sealer at 180°C for 5 sec. Proceed to thermal cycling. Use the appropriate thermal cycling conditions as specified in Table 8

▪ **For the ▪ QX200 AutoDG ddPCR System or ▪ QX600 AutoDG ddPCR System**, remove the droplet plate containing ddPCR droplets from the Automated Droplet Generator. Seal the plate using the PX1 PCR Plate Sealer at 180°C for 5 sec. Proceed to thermal cycling. Use the appropriate thermal cycling conditions as specified in Table 8

▪ **For the ▪ QX ONE ddPCR System**, thermal cycling is integrated into and sequentially performed by the system itself. No additional equipment or sample handling is required

Table 8. Cycling conditions.*

Cycling Step		Temperature, °C	Time	Number of Cycles	Ramp Rate
Hold (QX ONE ddPCR System only)		25	3 min	1	
Enzyme activation		95	10 min	1	
Denaturation		94	30 sec	40	
Annealing/extension		60	1 min		2°C/sec
Enzyme deactivation		98	10 min	1	
Hold	QX200 or QX600 ddPCR System	4	∞	1	
	QX ONE ddPCR System	25	1 min	1	

* For the C1000 Touch Thermal Cycler, use a heated lid set to 105°C and set the sample volume to 40 µl.

Data Acquisition

Follow the instructions for data acquisition based on the system in use. Follow local environmental health and safety guidance for appropriate disposal of materials after droplet reading.

Note: The Vericheck ddPCR RCAAV Kit is compatible with the Vericheck ddPCR Replication Competent AAV Assay Protocol File, which streamlines data acquisition and facilitates viewing and analysis of targeted data. The Assay Protocol File (APF) is compatible with QX Manager Software Premium Edition version 2.1 or higher for the QX200 and QX600 Systems. For more details or for instructions on using an APF, skip to the Using an Assay Protocol File section, beginning on page 9.

For the ■ QX200 ddPCR System and the ■ QX200 AutoDG ddPCR System:

The Vericheck ddPCR RCAAV Kit is compatible with QX Manager Software v1.2 or higher (all editions). Refer to the QX Manager Software Standard Edition (10000107223), Premium Edition (1000153878), or Regulatory Edition (10000107224) User Guide for detailed information on QX200 instrument and plate setup.

1. Place the sealed 96-well plate in the droplet reader.

2. When setting up a new plate template, first select the wells to include/exclude under the Exclude tab.

3. Move to the Edit tab and designate the following (see Figure 3 for details):

   - Experiment Type: Direct Quantification (DQ)

   - Sample Descriptions: Determined by user

   - Sample Type: Determined by user (positive control wells should be designated as Pos Ctrl)

   - Supermix: ddPCR Supermix for Residual DNA Quantification

   - Assay Type: Single Target per Channel

   - Target Name(s): Determined by user

   - Target Type: Unkn

   - Signal Ch1: FAM

   - Signal Ch2: HEX

4. Press Apply and then Save the template.

5. Press Start Run.

For the ■ QX600 AutoDG ddPCR System:

The Vericheck ddPCR RCAAV Kit is compatible with QX Manager Software Standard Edition v2.0 and Premium Edition v2.1 or higher. Refer to the QX Manager Software Standard Edition (10000153877) or Premium Edition (10000153878) User Guide for detailed information on QX600 instrument and plate setup.

1. Place the sealed 96-well plate in the droplet reader.

2. When setting up a new plate template, first select the wells to include/exclude under the Exclude tab.

3. Move to the Edit tab and designate the following (see Figure 3 for details):

   - Experiment Type: Direct Quantification (DQ)

   - Sample Descriptions: Determined by user

   - Sample Type: Determined by user (positive control wells should be designated as Pos Ctrl)

   - Supermix: ddPCR Supermix for Residual DNA Quantification

   - Assay Type: Single Target per Channel

   - Target Name(s): Determined by user

   - Target Type: Unkn

   - Signal Ch1: FAM

   - Signal Ch2: HEX

4. Press Apply and then Save the template.

5. On the Run Setup screen, name the plate and select the supermix. Press Start Run.

For the ■ QX ONE ddPCR System:

The Vericheck ddPCR RCAAV Kit is compatible with QX ONE Software v1.2 or higher (all editions). Data acquisition is integrated into and sequentially performed by the system itself. No additional equipment or sample handling is required.
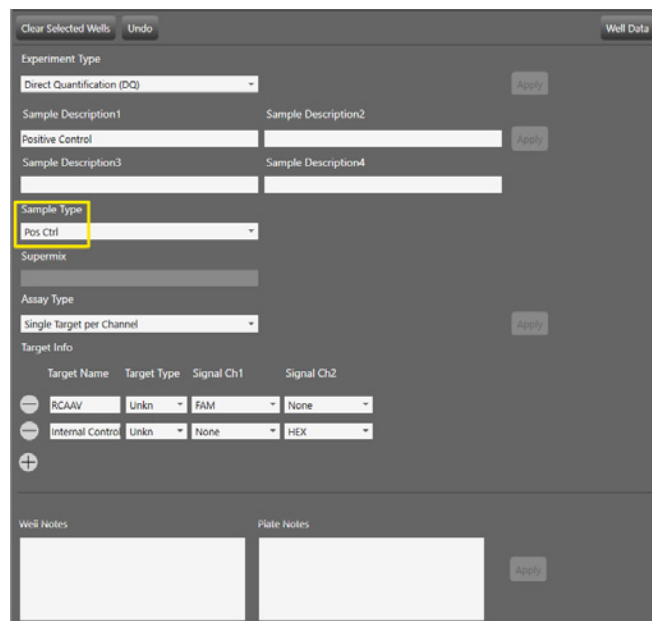

Fig. 3. Example of plate setup in the Plate Editor screen. Positive control wells should have the sample type Pos Ctrl (yellow rectangle).

Data Analysis: Thresholding and Quality Check

Positive Control–Based Autothresholding

Positive control–based autothresholding generates thresholds for each channel based on the wells marked as having the sample type Pos Ctrl. The software then applies those thresholds to all currently selected wells. QX Manager or QX ONE Software version 1.2 or higher (all editions) is required for positive control–based autothresholding. Reference the user guide for the software in use for detailed instructions about data analysis. See the Positive Control–Based Manual Thresholding section for software versions that do not support positive control–based autothresholding.

To threshold a plate with one assay:

1. Ensure that only the positive control wells are marked as the sample type Pos Ctrl. If necessary, navigate to the Plate Editor tab and select the positive control wells. Under Sample Type, use the dropdown menu to select Pos Ctrl and click Apply. See Figure 3 for an example of the Plate Editor screen.

2. Navigate to the 2D Amplitude tab. Select all wells, including control wells.

3. Click the Auto with Tilt button, select Positive Control Wells at the bottom of the dropdown menu, and click OK. This will autothreshold all selected wells based on the designated positive control wells. See Figure 4 for details.

4. To view the autothreshold lines that have been applied to the wells, click the red or magenta SD crosshairs tool.

Important: At least one of the selected wells must have the sample type Pos Ctrl in order to perform this method of thresholding.

To threshold a plate with multiple assays:

If multiple assays are run on the same plate, the wells for each assay must be thresholded separately. The positive control wells from only one assay should be marked with the sample type Pos Ctrl at a time. Positive control wells from all other assays on the plate should be marked as the sample type Unknown.

1. Navigate to the Plate Editor tab and use the Sample Type dropdown menu to mark the positive control wells for the first assay **only** as Pos Ctrl and click Apply.

2. Ensure positive control wells for all additional assays are marked as the sample type Unknown.

3. Navigate to the 2D Amplitude tab and select the wells for the first assay, including the control wells.

4. Click the Auto with Tilt button, select Positive Control Wells at the bottom of the dropdown menu, and click OK.

5. Return to the Plate Editor tab and change the positive control wells for the first assay to the sample type Unknown. Click Apply to save the changes.

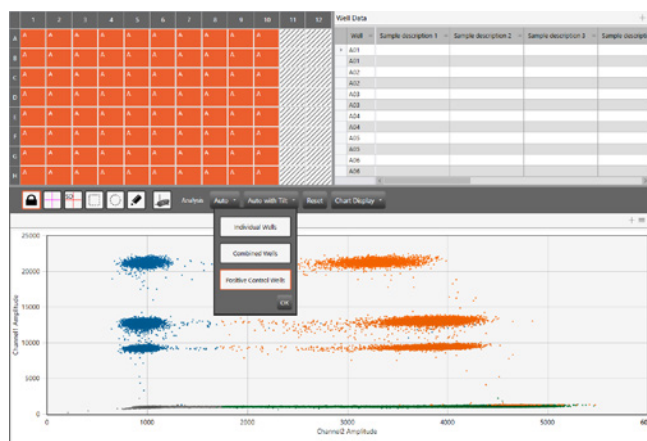6. Repeat steps 1–5 until the wells for all assays have been thresholded.

Fig. 4. 2D Amplitude screen when using positive control–based autothresholding with tilt.

Positive Control–Based Manual Thresholding

To threshold manually, follow the steps below.

1. Navigate to the 2D Amplitude tab. Select the wells of interest on the plate, including the control wells.

2. Click the Auto with Tilt button and select Combined Wells at the bottom of the dropdown menu. This will tilt correct all selected wells.

3. Select the positive and negative control wells for the assay. Manually threshold the control wells using the pink manual thresholding tool on the 2D Amplitude tab.

4. Copy the manual threshold values applied to the control wells and paste it onto all other wells (the sample wells).

Tip: Refer to the Droplet Digital PCR Applications Guide (bulletin 6407) for detailed instructions and best practices for setting manual thresholds.

Quality Check

Follow the steps below to check the data quality before proceeding with analysis. The quality metrics are summarized in Table 9.

1. Navigate to the Event Counts tab and evaluate the total droplet counts for all wells.

   Recommended best practice: If the total droplet count is <10,000 in any well, do not include the well in the final analysis. If all wells fail for a sample, refer to the Troubleshooting section.

2. Navigate to the 2D Amplitude tab and examine the control wells for uniformity. See Figure 5 for example positive and negative control well 2D plots. Do not use poor-quality positive control well(s) as thresholding controls (see Troubleshooting section for examples of poor-quality wells).

   Tip: If any poor-quality wells were used to threshold the plate, return to the Plate Editor tab, mark poor-quality positive control well(s) as having the sample type Unknown, and apply the change. Then, threshold the plate again using positive control–based autothresholding. Only one positive control well is required for accurate thresholding.

3. Examine the replicate sample wells for uniformity and examine any outliers for problems. RCAAV-positive samples are expected to contain only one set of FAM positive clusters. Do not include problem wells in the final analysis. If any clusters are cut off by the thresholds, the thresholds should be manually adjusted. See Figure 6 for an example sample well plot.
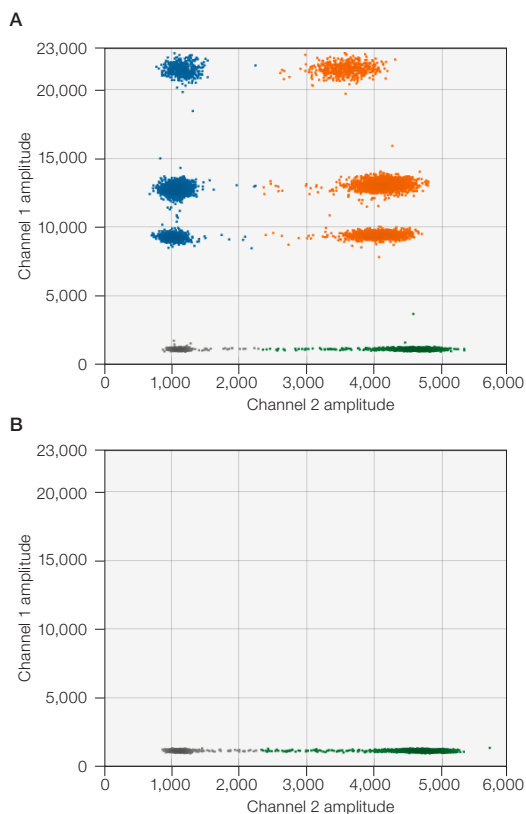
Fig. 5. Example 2D plots for assay controls. **A**, an example of a 2D plot for the positive control with 3 sets of FAM positive clusters. Positive control plots are expected to contain multiple FAM positive clusters. The separation between these clusters may vary such that the bottom two sets of clusters may overlap. An acceptable positive control 2D plot will have either 2 or 3 sets of FAM positive clusters. The plot shown is from 1 well. **B**, an example of a 2D plot for one negative control well. Negative control wells contain internal control and should have a HEX positive cluster.
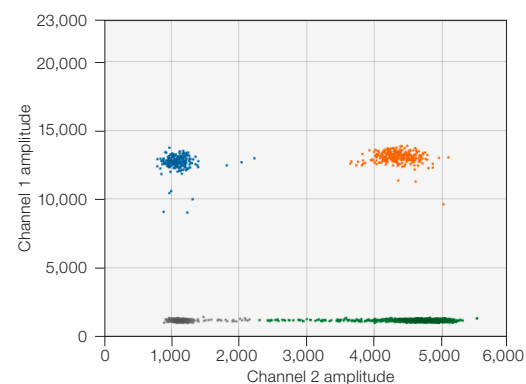
Fig. 6. Example 2D plot for one RCAAV-positive sample well. Sample plots are expected to contain only one set of FAM positive clusters. The amplitude of the positive clusters may vary depending on the serotype tested and the sample preparation method used.

4. Navigate to the Concentration tab and examine the control wells and each sample's replicate wells for uniformity. Positive control wells are expected to have a channel 1 (FAM) concentration of 262–1,947 copies/µl and a channel 2 (HEX) concentration of 800–1,200 copies/µl. Negative control wells are expected to have a FAM concentration of <0.12 copies/µl and a HEX concentration of 800–1,200 copies/µl. If positive control wells have the concentration value No Call and Status: CHECK, follow instructions in the Troubleshooting section to resolve the error.

5. On the Concentration tab, examine each sample's HEX concentration. The HEX concentration is expected to be between 800–1,200 copies/µl for all wells with ddPCR RCAAV Internal Control. A >20% difference in internal control concentration between control and sample wells may indicate PCR inhibition.

Table 9. Summary of quality metrics to check for each plate.

Metric	Applicable Well Type	Acceptable Result (Per 1 well)	Suggested Outcome for Failure
Accepted Droplet Count	All wells	≥10,000 droplets	Remove well from analysis
2D phenotype	Positive control wells	Expected, uniform 2D phenotype*	Do not use well as a thresholding control; remove well from analysis
2D phenotype	All wells	Expected, uniform 2D phenotype*	Remove well from analysis
Threshold locations	All wells	Thresholds do not cut through clusters	Manually adjust thresholds if they cut directly through clusters
Positive control concentration	Positive control wells	FAM: 262–1,947 copies/µl HEX: 800–1,200 copies/µl	If controls are not within expected range, fail plate
Negative control concentration	Negative control wells	FAM: <0.12 copies/µl HEX: 800–1,200 copies/µl	If controls are not within expected range, fail plate
Sample internal control concentration	Sample wells	HEX: 800–1,200 copies/µl	Remove well from analysis, dilute and retest sample

* Expected control well 2D phenotypes are shown in Figure 5 and an example positive sample phenotype is shown in Figure 6. See the Troubleshooting section for examples of well failures and unexpected 2D phenotypes.

Data Analysis: Result Interpretation
RCAAV Assay 2D Plot Cluster Interpretation
Table 10 defines each cluster of the 2D plots for the RCAAV assay. RCAAV is detected in FAM and the internal control signal is detected in HEX. Example positive control and negative control 2D plots are shown in Figure 5.

Table 10. RCAAV Assay 2D plot cluster interpretation.

Data Column Name	Product
Ch1+Ch2- (Blue)	RCAAV
Ch1-Ch2+ (Green)	Internal control
Ch1+Ch2+ (Orange)	RCAAV + internal control

Data Interpretation and Quantification
Sample concentrations should be used to determine if the sample is RCAAV positive. **Each well should be analyzed individually.** Table 11 provides an interpretation guide for calling a sample positive or negative, based on the positivity cutoff level of 0.12 copies/µl. The positivity cutoff level was determined based on contrived samples that do not contain residual Rep contamination. If desired, users can adjust the positivity cutoff level for their unique sample type.

Table 11. Vericheck ddPCR RCAAV Kit results interpretation guide.

Status	Channel 1 (FAM) Result	Interpretation
Negative	<0.12 copies/µl*	RCAAV not detected
Positive	≥0.12 copies/µl	RCAAV detected

* The concentration reported is copies/µl in the final 1x ddPCR reaction.

Using an Assay Protocol File
The Vericheck ddPCR RCAAV Kit is compatible with the Vericheck ddPCR Replication Competent AAV Assay Protocol File. The assay protocol file (APF) contains specific instructions and rules for qualitative and quantitative evaluations of ddPCR data. APFs are connected to a specific results feature in QX Manager Software that facilitates viewing and analysis of targeted data. When using the Vericheck ddPCR Replication Competent AAV APF, key assay results are summarized on the APF Results tab. QX Manager Software Premium Edition v2.1 or higher is required for use with APFs. The APFs are compatible with the QX200 and QX600 Systems.

Understanding APF Results
The results reported by the APF are summarized in Table 12. Results will be provided for each well individually.

Table 12. Explanation of results summarized by the APF.

Result	Metric
Replication Competent AAV Status	Positive or Negative*
Replication Competent AAV Concentration	FAM concentration, copies/µl
Internal Control Concentration	HEX concentration, copies/µl
Accepted Droplet Count	Total event count for the well

* See Table 13 for status determination details.

Replication Competent AAV Status is determined according to Table 13. Sample concentrations are used to determine whether the sample is RCAAV positive. Wells are called positive or negative using a positivity cutoff level of 0.12 copies/µl in the final 1x ddPCR reaction. The positivity cutoff level was based on contrived samples that do not contain residual Rep contamination. If desired, users can adjust the positivity cutoff level for their unique sample type by following the steps in the Adjusting APF Positivity Cutoff section on page 12.

Table 13. Replication competent AAV status determination.

Status	FAM Result	Interpretation
Negative	<0.12 copies/µl*	RCAAV not detected
Positive	≥0.12 copies/µl	RCAAV detected
Not Calculated	Undefined	If a concentration could not be determined by the software, an RCAAV status cannot be calculated

* Positivity status calls are made based on non-rounded concentration values.

Additionally, the APF provides warnings for the common data quality issues listed in Table 14. An example APF Result Summary Table for a problem well is shown in Figure 7. Users should also perform a quality check of their data.

Table 14. APF data quality warnings and detailed explanation.

Result	Warning Message	Reason for Warning
Accepted Droplet Count	CHECK: Below Droplet Count Range	Total event counts for the well <10,000 droplets*
Internal Control Concentration	CHECK: Below Target Range	HEX concentration <800 copies/µl
	CHECK: Above Target Range	HEX concentration >1,200 copies/µl

* It is recommended to exclude wells with <10,000 droplets from analysis. See the Troubleshooting section for details.

Fig. 7. Example of a data quality warning in the APF Result Summary table.

Importing an APF for Use in QX Manager Software

1. Launch QX Manager Premium Edition Software and navigate to the Template Setup tab.

2. Click APF Management.

3. Click Import Package, select Vericheck_RCAAV_QX200.apfpack or Vericheck_RCAAV_QX600.apfpack from the File Explorer window, and click Open to import the APF.

4. APFs can also be deleted from this tab by checking the box next to an APF and clicking Delete Package.

See Figure 8 for a summary of steps.
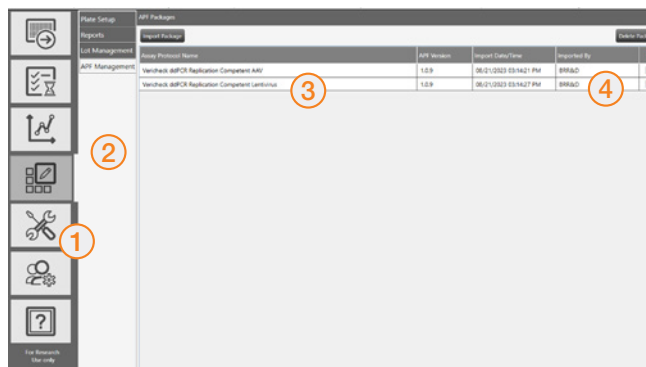
Fig. 8. Steps to import an APF package into QX Manager Software Premium Edition.

Data Acquisition Using an APF

1. Navigate to the Add Plate tab, click Add Plate, and add plate with thermal cycled droplets to the instrument.

2. Click Configure Plate.

3. In the Choose APF dropdown menu, select the Vericheck ddPCR Replication Competent AAV APF (Figure 9).
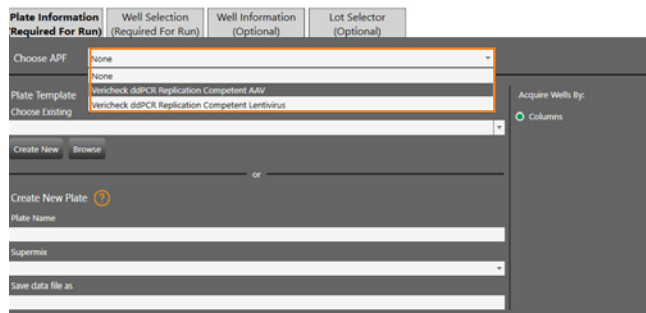
Fig. 9. Selecting an APF for use.

4. Under the Plate Template heading, either choose an existing template or set up a new plate template.

Note: To use an existing template, that template must have been generated using the same APF package version as the package that was selected from the dropdown menu.

5. To set up a new plate template, follow the steps below:

   a. Select the wells to include/exclude under the Exclude tab.

   b. Move to the Edit tab (Figure 10) and designate the following:

      ▪ Well Type: RCAAV

      ▪ Sample ID: Determined by user (every well **must** have a unique Sample ID)

      ▪ Sample Descriptions: Determined by user

      ▪ Sample Type: Determined by user (positive control wells **must** be designated as Pos Ctrl)

c. All other fields will automatically be filled in with the appropriate settings for the Vericheck ddPCR RCAAV Kit.

d. Press Apply and then Save the template.

Important: Each well should be analyzed individually. When setting up or editing a Plate Template, every well **must** have a unique Sample ID to ensure accurate APF results. Replicate wells can share the same Sample Descriptions but must have different Sample IDs.

6. Press Start Run.

Fig. 10. Example of plate setup in the Plate Editor screen. To ensure accurate analysis of results, each well must have a unique Sample ID. Positive control wells must have the sample type Pos Ctrl.

Data Analysis Using an APF
2D Plots

1. Navigate to the 2D Amplitude tab and select all wells, including the control wells. Click the Auto with Tilt button, select Positive Control Wells at the bottom of the dropdown menu, and click OK. This will tilt correct all the wells to ensure accurate thresholding based on the positive controls.

2. Examine the control wells for uniformity. Do not use poor quality positive control well(s) as thresholding controls.

Tip: If any poor-quality wells were used to threshold the plate, return to the Plate Editor tab, mark poor-quality positive control well(s) as having the sample type Unknown, and apply the change. Then, threshold the plate again using positive control–based autothresholding. Only one positive control well is required for accurate thresholding.

3. Examine the replicate sample wells for uniformity and examine any outliers for problems. Refer to the Troubleshooting section for descriptions of common failure modes. Do not include problem wells in the final analysis. If any clusters are cut off by the thresholds, the thresholds should be manually adjusted.

APF Results

Follow the steps below to view the APF results, perform a quality check, and export results. **Each well should have a unique Sample ID and should be analyzed individually.**

1. Navigate to the APF Results tab in QX Manager Software and select a well to view the APF Result Summary (Figure 11).
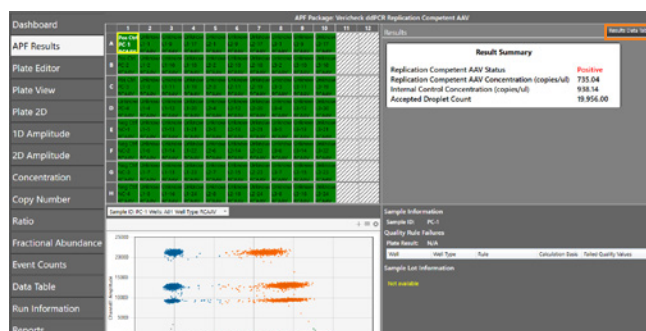
Fig. 11. APF Results tab showing results for a positive control well. Orange rectangle shows the Results Data Table button.

2. On the APF Results tab, the results for all wells can be viewed together by clicking Results Data Table in the upper right corner of the screen (Figure 11, orange rectangle). All concentration values in the Results Data Table are given in copies/µl of the final 1x ddPCR reaction.

3. Review the quality metrics summarized in Table 9 prior to continuing with analysis.

4. To export a .csv file containing the APF results, click on Import/Export in the top left portion of the screen. Next, click Export Sample Results(.csv) (Figure 12).
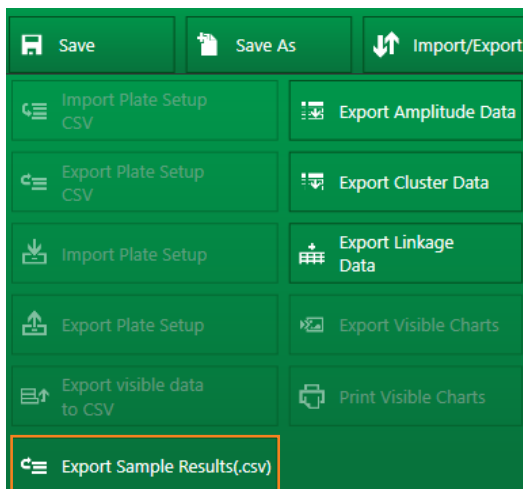
Fig. 12. Screenshot of the APF result export process. Click Export Sample Results(.csv) (orange rectangle) to export APF results.

5. To export a report, navigate to the Reports tab, ensure APF Results is checked to be included in the report (Figure 13), and click Generate Report. The Result Summary Table will be exported as part of the report.
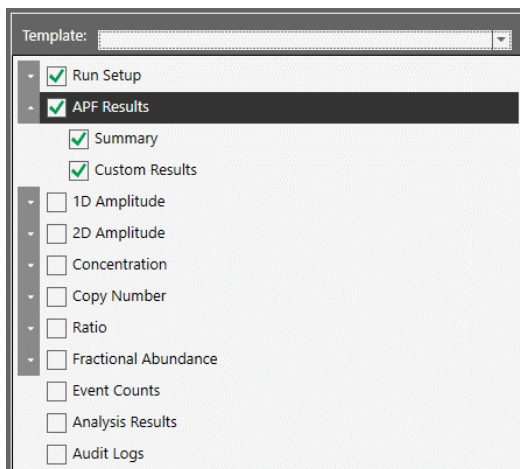

Fig. 13. On the Reports tab, ensure APF Results is checked prior to generating the report.

Adjusting APF Positivity Cutoff

Each well is called positive or negative using a default positivity cutoff level of 0.12 copies/µl. This means that any well with ≥0.12 copies/µl will be called positive and any well with <0.12 copies/µl will be called negative. Positivity calls are made on non-rounded concentration values. When adjusting the positivity cutoff, users should enter a concentration value in copies/µl of the final 1x ddPCR reaction. This value will replace 0.12 when the APF determines RCAAV status.

1. From the QX Manager Software main window, navigate to the Template Setup 🖼 tab.

2. Click APF Management.

3. Click on the Vericheck ddPCR Replication Competent AAV APF package name and more details will open below it.

4. In the Variables table, click the bolded number under the Modified column header and type in the desired positivity cutoff, in copies/µl of the final 1x ddPCR reaction (Figure 14).
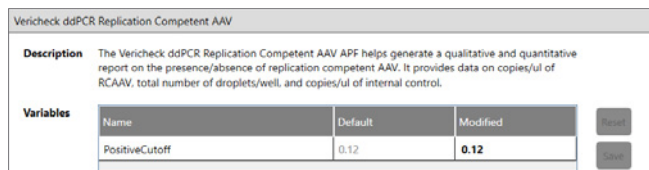
Fig. 14. Vericheck ddPCR Replication Competent AAV APF package details in the APF Management tab of QX Manager Software. The positivity cutoff for the APF can be adjusted by typing a new number into the Modified box of the Variables table.

5. Click Save. For all future plates, the modified value will be used as a cutoff for calling wells RCAAV positive.

Note: The positivity cutoff level of 0.12 copies/µl was determined using contrived samples that do not contain residual Rep contamination. Users can adjust the APF custom positivity cutoff by determining their own positivity cutoff based on their sample and experimental testing.

Troubleshooting

This section lists some common failure modes with their phenotypes, descriptions, and suggested resolutions. For a complete list of failure modes, refer to the Droplet Digital PCR Applications Guide (bulletin 6407) and the instruction manual of the instrument.

Droplet Max Amplitude Error

Problem: After all wells have been autothresholded, some control wells may have the error Droplet Maximum Amplitude Exceeded. Wells with this error will have No Call as the concentration and CHECK as the Status (see Figure 15). Positive control wells with this error will still apply accurate thresholds to the entire plate, but the concentration for wells with this error will not be visible until it has been resolved.

Fig. 15. Wells with Droplet Maximum Amplitude Exceeded error will have No Call as the concentration (cyan oval) and CHECK as the status.

Resolution: On the 2D Amplitude tab, select all wells with the Droplet Maximum Amplitude Exceeded error. Then, click the red SD crosshairs tool to show the autothreshold numbers (Figure 16, cyan oval). Click inside one of the threshold boxes (Figure 16, purple oval) and press enter to accept the thresholds manually. The threshold values will not change, but the software will now display a concentration for those wells and have the status Manual (Figure 17).
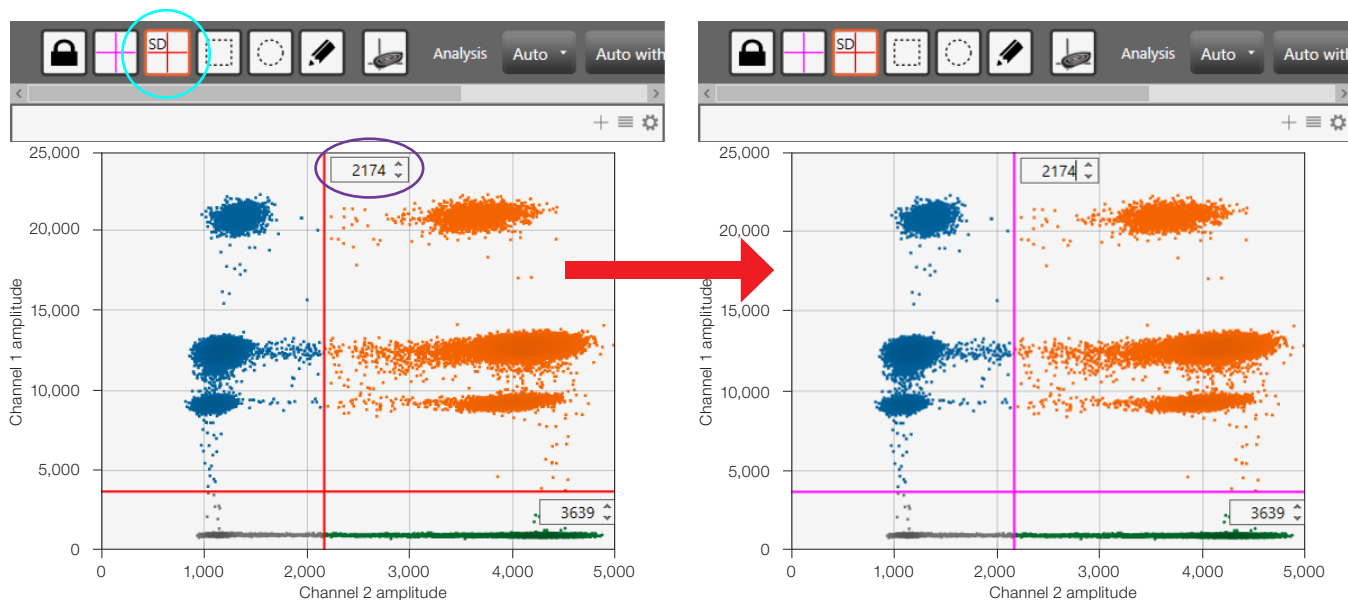

Fig. 16. Steps to resolve the Droplet Maximum Amplitude Exceeded error. Cyan oval is the red SD crosshairs tool and purple oval is the threshold box. After resolving the error, the threshold numbers will remain the same, but the thresholding lines will turn from red to magenta, indicating that the thresholds have been manually accepted.

Conc(copies/µL)	Molecular Weight(pg/µL)	Status	Status Reason	Experiment
898		Manual	Droplets Analyzed Manually	RDQ
623		Manual	Droplets Analyzed Manually	RDQ
944		Manual	Droplets Analyzed Manually	RDQ
641		Manual	Droplets Analyzed Manually	RDQ
923		Manual	Droplets Analyzed Manually	RDQ
652		Manual	Droplets Analyzed Manually	RDQ
900		OK		RDQ
0		OK		RDQ
915		OK		RDQ
0		OK		RDQ
943		OK		RDQ
0		OK		RDQ

Fig. 17. Data table after the Droplet Maximum Amplitude Exceeded error has been resolved.

No Negative Droplets

Problem: Only high-amplitude clusters are present in sample well (Figure 18). The sample is too concentrated and is outside of the dynamic range.

Resolution: Exclude well from analysis, dilute sample, and test again.
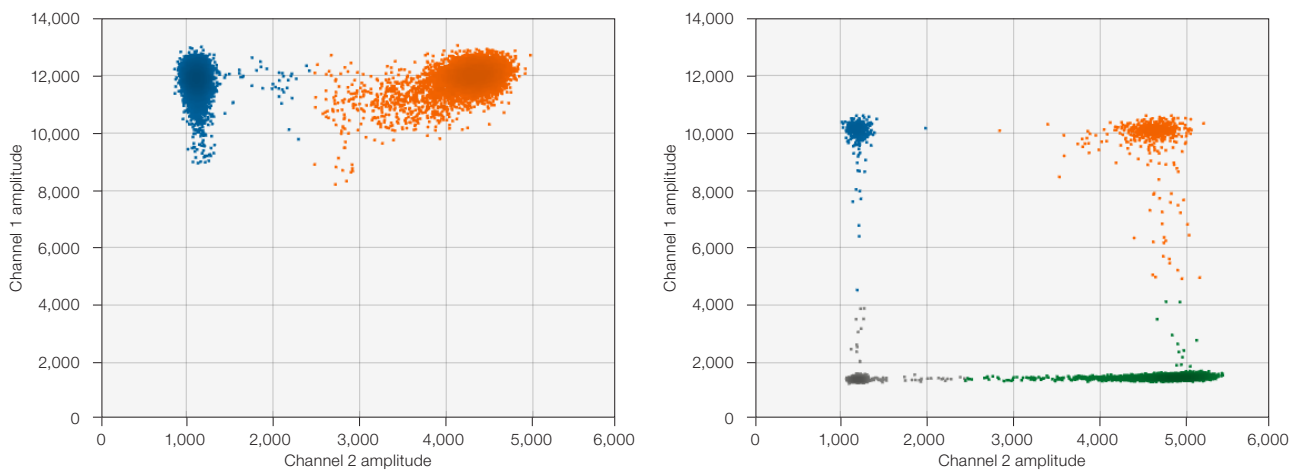
Fig. 18. No negative droplets. Problem sample well (left) versus normal sample well (right).

No or Low Total Droplet Counts in Sample Wells Only

Problem: Multiple replicate sample wells have a droplet count <10,000 (Figure 19). If all control wells have droplet counts >10,000, the issue is likely caused by inhibitors carried over from the starting matrix or sample preparation.

Resolution: Exclude sample wells with low droplet counts from analysis. If all replicate sample wells have low total droplet counts, dilute the sample to decrease inhibitor concentration and repeat Droplet Digital PCR. Alternatively, further purify the DNA sample to remove PCR inhibitors.
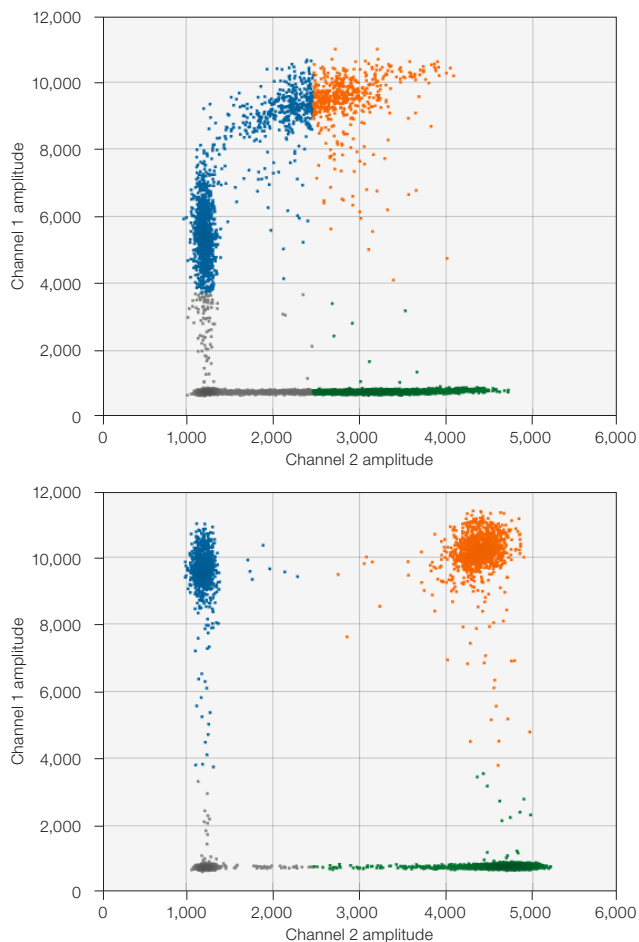
No or Low Total Droplet Counts in Both Sample and Control Wells

Problem: Sample and control wells have droplet counts <10,000 (Figure 19). If control wells have low droplet counts, inhibition is not the likely cause of the low counts.

Resolution: Exclude wells with low total droplet counts from analysis. For the QX200 or QX600 Systems, repeat Droplet Digital PCR and allow the plate to hold at the final 4°C step for at least 30 minutes prior to droplet reading to increase droplet numbers. If the problem persists, contact Technical Support.
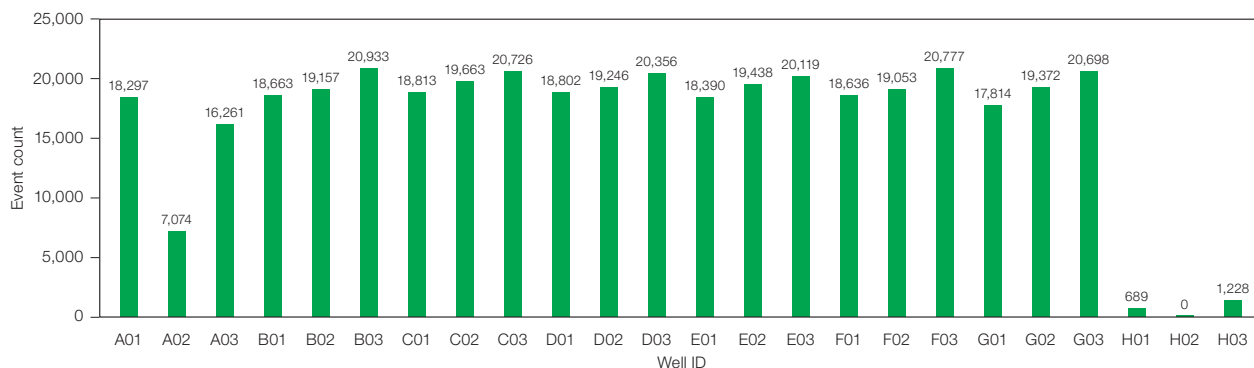
Fig. 19. Event count graph in which some wells have a total droplet count <10,000.

PCR Inhibition

Problem: Separation between clusters decreases and rain increases (Figure 20). Droplet counts may also be higher or lower than expected.

Resolution: If a concentration cannot be calculated, exclude the well from analysis. Dilute the sample to decrease inhibitor concentration and repeat Droplet Digital PCR. Alternatively, further purify the DNA sample to remove PCR inhibitors.

Mirroring

Problem: Droplets exhibit two or more distinct sizes (Figure 21). This indicates a potential consumable failure or particulates from samples, environment, tips, or reagents.

Resolution: Exclude the well from analysis and repeat Droplet Digital PCR, preferably with a different lot of droplet generation consumables.

Fig. 20. PCR inhibition. Problem sample well (top) versus normal sample well (bottom).
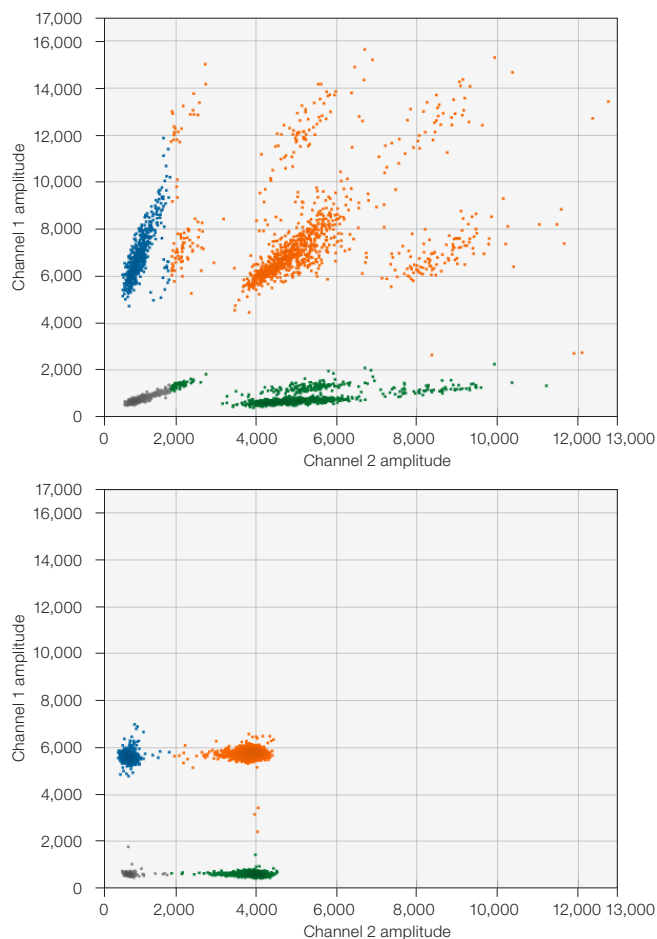
Fig. 21. Mirroring. Problem sample well (top) versus normal sample well (bottom).

Droplet Shredding

Problem: Shredded droplets appear on the diagonal through the negative droplet cluster (Figure 22).

Resolution: Exclude well from analysis or repeat Droplet Digital PCR.
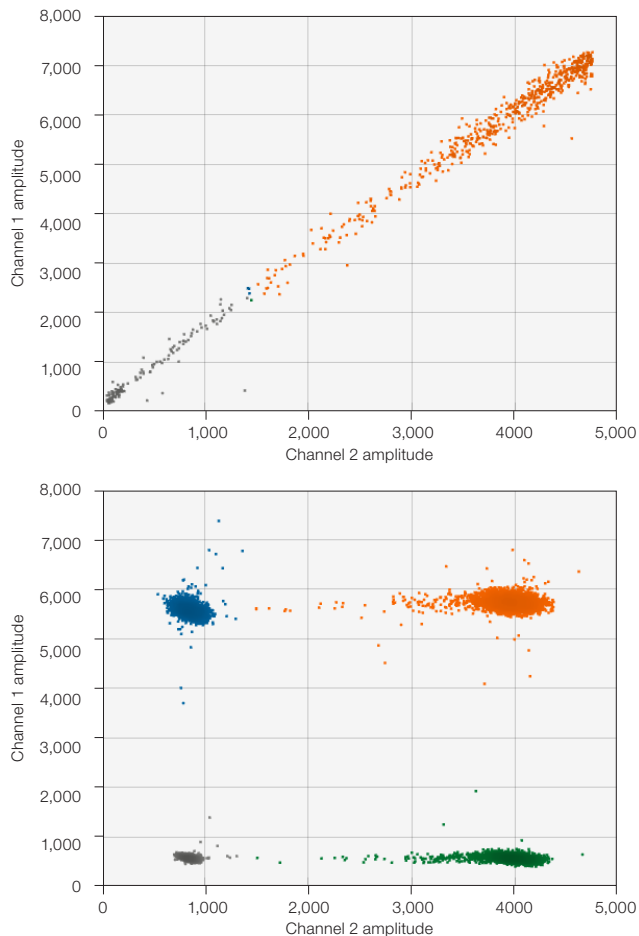
Fig. 22. Droplet shredding. Problem sample well (top) versus normal sample well (bottom).

Incorrect Autothresholding

Problem: Droplets are incorrectly thresholded using autothresholding (Figure 23).

Resolution: Ensure that wells were thresholded using the positive control–based autothresholding with tilt. If this does not resolve the issue, follow instructions for manual thresholding. If necessary, refer to the ddPCR Applications Guide (bulletin 6407) for best practices setting manual thresholds.
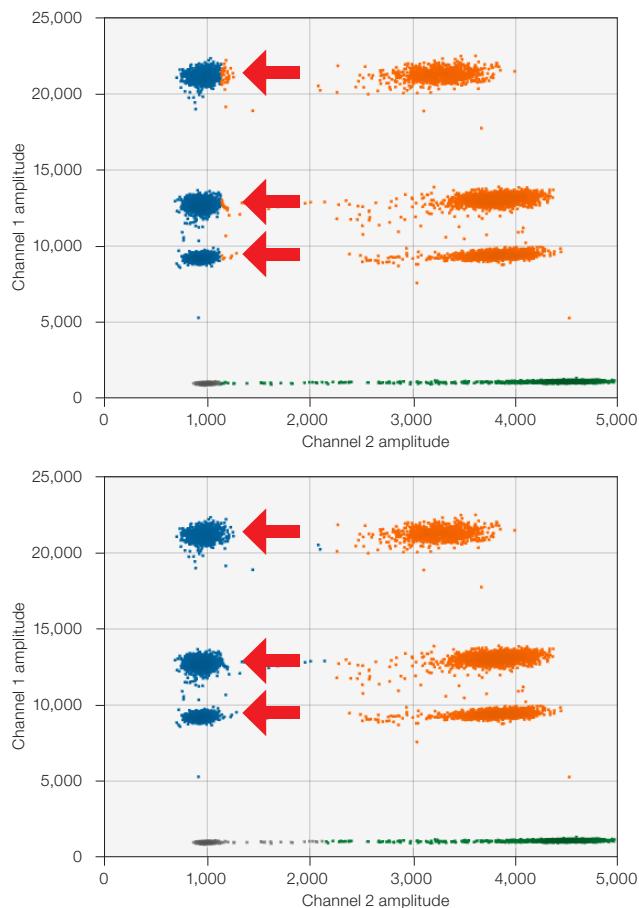
Fig. 23. Incorrect autothresholding. Incorrect thresholding indicated by arrows (top) versus correct thresholding after manual adjustment (bottom).

Visit **bio-rad.com/ddPCR-Vericheck-RCAAV** for more information.