

VeriCheck ddPCR™ Replication Competent Lentivirus Kit User Guide

Catalog #	Description
12019529	VeriCheck ddPCR Replication Competent Lentivirus 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 a lentiviral 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 Lentivirus (RCL) Kit can be used to confirm the presence or absence of replication competent lentivirus during the production of cell and gene therapy drug products.

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 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 MseI restriction enzyme. The maximum allowable concentrations of common inhibitors in the starting sample prior to extraction are listed in Table 2. Samples were extracted using the QIAGEN QIAamp DNA Mini Kit.

Table 1. Analytical performance summary.

Dynamic Range	0.35 to 7,500 copies/µl*
Linearity	Slope = 1.00, R ² = 1.00
LOD	0.35 copies/µl*
LOQ	0.50 copies/µl*
LOB	0.00 copies/µl*
Specificity	No detection of HEK293, CHO, Human, <i>E. coli</i> , or <i>Mycoplasma</i> genomic DNA

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

Table 2. Inhibitor tolerance: maximum allowable concentrations of inhibitor per starting sample, prior to extraction.

Inhibitor	Allowable Concentration in Sample
DMEM complete media	90%
Supernatant	90%
Clarified cell lysate	90%
97.5% PBS + 2.5% HSA	90%
Tween-20	1%
Pluronic F-68	0.1%
PEI	1 pg/µl
DMSO	10%
Triton X-100	1%
NP-40	1%
EDTA	2 mM
SDS	1%

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 RCL Internal Control (20,000 copies/µl)	1	0.90 ml
ddPCR RCL Positive Control	1	0.08 ml
Nuclease-Free Water (Negative Control)	1	1.50 ml
ddPCR RCL Assay (20x)	1	0.15 ml

Table 4. Additional materials required.*

System	Instruments	Consumables and Reagents
QX200™ Droplet Digital™ PCR System	<ul style="list-style-type: none"> 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) 	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> Automated Droplet Generator (#1864101) QX200 Droplet Reader (#1864003) C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module (#1851197) PX1 PCR Plate Sealer (#1814000) 	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> QX ONE Droplet Digital PCR System (#12006536) PX1 PCR Plate Sealer (#1814000) 	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> Automated Droplet Generator (#1864101) QX600 Droplet Reader (#12013328) C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module (#1851197) PX1 PCR Plate Sealer (#1814000) 	<ul style="list-style-type: none"> 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 (extraction)	<ul style="list-style-type: none"> See extraction kit for details 	<ul style="list-style-type: none"> Recommended: QIAGEN QIAamp DNA Mini Kit (QIAGEN, #51304)

* 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

Perform DNA extraction for each sample with a QIAGEN QIAamp DNA Mini Kit following QIAGEN [Appendix J: Protocol for Crude Cell Lysates and Other Samples](#) or with a user-preferred extraction method. For optimal performance, samples extracted with the QIAamp Kit should be eluted in 200 µl. Table 2 contains the maximum allowable concentrations of common inhibitors in the starting sample, prior to extraction.

Samples with high levels of unfragmented genomic DNA (including non-RCL DNA) may require restriction enzyme digestion, as unfragmented DNA can act as an inhibitor. Samples with >66 ng/well of total DNA should be digested using a restriction enzyme. The VeriCheck ddPCR RCL Kit is compatible with MseI restriction enzyme. Samples with >1 µg/well of total DNA should be diluted prior to testing.

Tip: Samples can be digested prior to Droplet Digital PCR or digested directly in droplets by adding 0.5 µl MseI (at 10,000 units/ml) to each 20 µl ddPCR reaction. For direct digestion, reduce the volume of water added to the sample wells to account for the addition of restriction enzyme and ensure a total volume of 20 µl per reaction.

Usage of Internal Control for Extraction Efficiency

Optional: While designed as an internal control for ddPCR assay performance, the ddPCR RCL Internal Control can also be spiked into the sample prior to extraction to serve as a control for extraction efficiency. The percentage recovery of the internal control in the extracted sample is a proxy indicator of the extraction efficiency.

The internal control should be added to each sample just prior to beginning extraction.

The volume of internal control added is variable and based on the final elution volume of the extracted sample. For a 200 µl elution volume, 25 µl of internal control should be added to the sample prior to extraction. The following equation can be used to calculate the volume of internal control to add to the sample for any elution volume:

$$V = E / 8$$

For example, for a 200 µl elution volume:

$$V = 200 \mu\text{l} / 8 = 25 \mu\text{l}$$

Where:

V = volume of internal control to spike into the sample prior to extraction, µl

E = elution volume, µl

8 refers to the volume of sample added to the 20 µl ddPCR reaction well, in µl

Plate Setup

An example plate setup is shown in Figure 1.

	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. 1. 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: Extracted DNA sample, which may include the internal control spiked in prior to extraction, is added to the ddPCR reaction. The RCL signal is read in channel 1 (FAM) and the internal control signal is read in channel 2 (HEX).

Pos Ctrl (ddPCR RCL Positive Control): Instead of sample, positive control is added to the ddPCR reaction. The positive control signal is read in channel 1 (FAM). The internal control is also added directly to the ddPCR reaction. The internal control signal is read in channel 2 (HEX).

Neg Ctrl (Nuclease-Free Water): Instead of sample, nuclease-free water is added to the ddPCR reaction. The internal control is also added directly to the ddPCR reaction. The internal control signal is read in channel 2 (HEX).

Reaction Setup

Table 5. Preparation of master mix (supermix and RCL assay mix).

Components	1x Reaction (µl)	48x Reactions + Overage* (µl)
ddPCR Supermix for Residual DNA Quantification	10	680
ddPCR RCL Assay	1	68
Total volume	11	748

* 14 samples + 1 positive control + 1 negative control = 16 total × 3 replicates each = 48 total reactions. Here, the master mix is prepared with 44 µl overage. It is recommended to prepare the master mix with ~40–50 µl overage.

Table 6. Preparation of control reaction mixes.

Components	1x Reaction (µl)	3x Reactions + Overage* (µl)
Master mix (From Table 5)	11	44
ddPCR RCL Positive Control or Nuclease-Free Water (Negative Control)	8	32
ddPCR RCL Internal Control	1	4
Total volume	20	80

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

Table 7. Preparation of sample reaction mix.

Components	1X Reaction (µl)	3x Reactions + Overage* (µl)
Master mix (from Table 5)	11	44
Sample	8	32
Nuclease-free water**	1	4
Total volume	20	80

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

** If ddPCR RCL Internal Control was **not** already added to the sample as part of the extraction process, it can be added here instead of nuclease-free water.

Procedure

- 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 1.
- 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.
- Ensure samples are thawed to room temperature and thoroughly mixed.
- Using Table 5, assemble master mix containing supermix and ddPCR RCL Assay. Vortex the master mix thoroughly at maximum speed for 15 sec.
- Prepare reaction mix for each set of three replicate wells by combining master mix (from step 4) with the controls or with the sample and water.
 - Using Table 6, prepare the negative control and positive control reaction mixes. Internal control must also be added to both control well reaction mixes.
 - Using Table 7, prepare the sample reaction mixes. Water is added to sample reaction mixes if internal control was already added to the sample prior to extraction.
 - 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 pipet.

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 described below 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):

- 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.
- Add 20 µl of 1x buffer control to any unused wells from which droplets will be generated.
- Load 70 µl of Droplet Generation Oil for Probes into the oil wells. For detailed instructions, refer to the QX200 Droplet Generator Instruction Manual (D094813).

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

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

- Load 20 µl of each reaction mix into the wells of a GCR96 Cartridge.
- Droplet generation occurs in sets of two columns (for example, droplets for columns 1 and 2 are generated simultaneously). Add 20 µl of buffer control to any unused wells in each set of columns from which droplets will be generated.
- 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.

- 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 RCL 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 2 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 pipet. 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	2°C/sec
Enzyme activation		95	10 min	1	
Denaturation		94	30 sec	40	
Annealing/extension		60	1 min		
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 RCL Kit is compatible with the VeriCheck ddPCR Replication Competent Lentivirus 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 v2.1 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 8.

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

The VeriCheck ddPCR RCL Kit is compatible with QX Manager Software v1.2 or higher (all editions). Refer to the QX Manager Software Standard Edition (10000107223), Premium Edition (10000153878), or Regulatory Edition (10000107224) User Guide for detailed information on QX200 instrument and plate setup.

- Place the sealed 96-well plate in the droplet reader.
- When setting up a new plate template, first select the wells to include/exclude under the Exclude tab.
- Move to the Edit tab and designate the following (see Figure 2 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

- Press Apply and then Save the template.
- On the Run Setup screen, name the plate and select the supermix. Press Start Run.

For the ■ QX600 AutoDG ddPCR System:

The VeriCheck ddPCR RCL Kit is compatible with QX Manager Software Standard Edition v2.0 or higher 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.

- Place the sealed 96-well plate in the droplet reader.
- When setting up a new plate template, first select the wells to include/exclude under the Exclude tab.
- Move to the Edit tab and designate the following (see Figure 2 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
- Press Apply and then Save the template.
- On the Run Setup screen, name the plate and select the supermix. Press Start Run.

For the ■ QX ONE ddPCR System:

The VeriCheck ddPCR RCL 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.

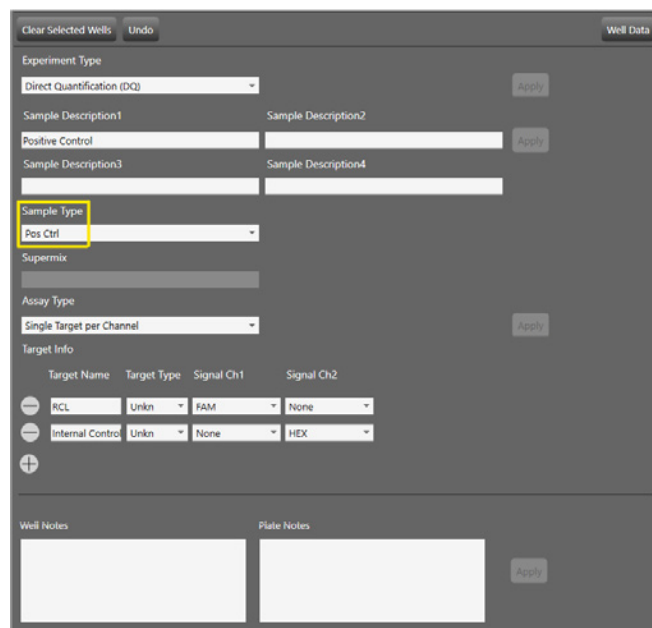


Fig. 2. Example of plate setup in the Plate Editor screen. Positive control wells should have the sample type Pos Ctrl (yellow rectangle).

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 applies the 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 for the assay are marked as 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 2 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 3 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 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.

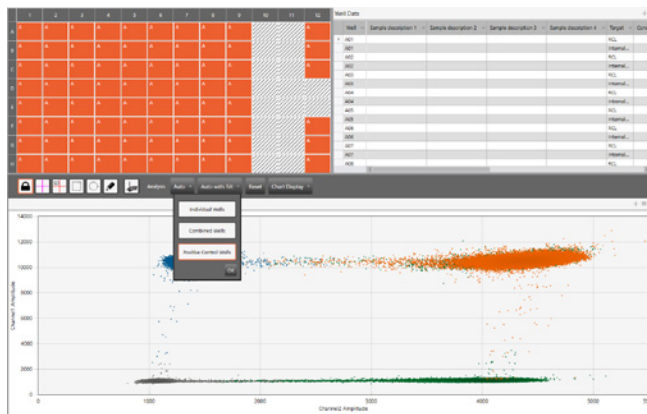


Fig. 3. 2D Amplitude screen using positive control–based autothresholding with tilt.

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 them 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 4 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.

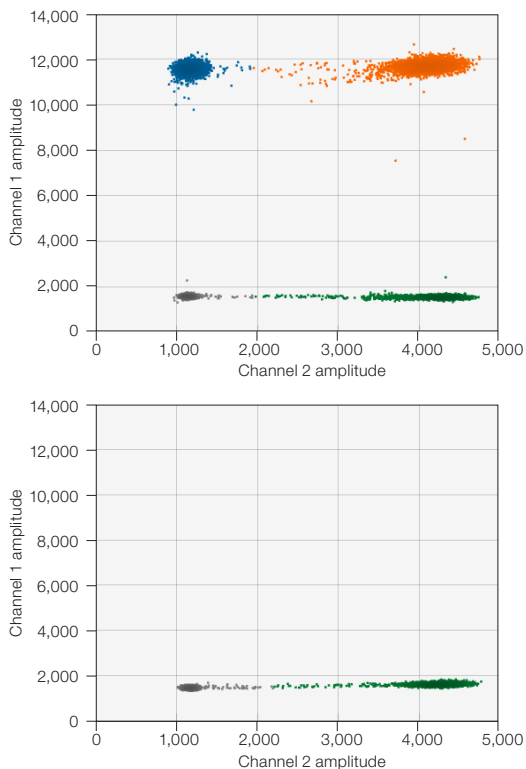


Fig. 4. Example 2D plots for the positive control (top) and negative control (bottom). Each plot is 1 well.

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.
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.

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

* Expected 2D phenotypes are shown in Figure 4. See troubleshooting section for examples of well failures and unexpected 2D phenotypes.

Data Analysis: Result Interpretation

RCL Assay 2D Plot Cluster Interpretation

Table 10 defines each cluster of the 2D plots for the RCL assay. RCL is detected in FAM and the internal control is detected in HEX. Example positive control and negative control 2D plots are shown in Figure 4.

Table 10. RCL assay 2D plot cluster interpretation.

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

Data Interpretation and Quantification

- Sample FAM concentrations should be used to determine if the sample is RCL 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 VSV-G contamination. If desired, users can adjust the positivity cutoff based on their sample and experimental testing.

Table 11. VeriCheck ddPCR RCL Kit results interpretation guide.

Status	FAM	Interpretation
Negative	<0.12 copies/μl*	RCL not detected
Positive	≥0.12 copies/μl	RCL detected

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

- Examine the HEX concentration of each sample, the positive control, and the negative control. The following formula can be used to determine the percentage recovery of each extracted sample:

$$R = (S/C) \times 100\%$$

Where:

R = percentage recovery

S = sample well HEX concentration, copies/μl

C = average positive control and negative control well HEX concentration, copies/μl

Using an Assay Protocol File

The VeriCheck ddPCR RCL Kit is compatible with the VeriCheck ddPCR Replication Competent Lentivirus 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, which facilitates viewing and analysis of targeted data. When using the VeriCheck ddPCR Replication Competent Lentivirus APF, key assay results are summarized on the APF Results tab. QX Manager Software Premium Edition version 2.1 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 described in Table 12. Results will be provided for each well individually.

Table 12. Explanation of results summarized by the APF.

Result	Metric
Replication Competent Lentivirus Status	Positive or Negative*
Replication Competent Lentivirus 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 Lentivirus Status is determined according to Table 13. Sample FAM concentrations are used to determine if the sample is RCL-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 VSV-G 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 11.

Table 13. Replication competent lentivirus status determination.

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

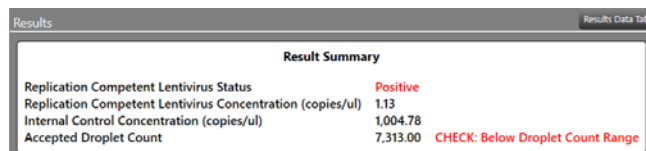
*Positivity status calls are made based on non-rounded concentration values.

Additionally, the APF provides a warning for a common data quality issue (see Table 14). An example APF Result Summary Table for a problem well is shown in Figure 5. 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*

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



Result Summary	
Replication Competent Lentivirus Status	Positive
Replication Competent Lentivirus Concentration (copies/ul)	1.13
Internal Control Concentration (copies/ul)	1,004.78
Accepted Droplet Count	7,313.00 CHECK: Below Droplet Count Range

Fig. 5. Example of an APF Result Summary table for a problem well. The data quality warning is shown on the right side of the table.

Importing an APF for Use in QX Manager Software

- Launch QX Manager Software Premium Edition and navigate to the Template Setup tab.
- Click APF Management.
- Click Import Package and select VeriCheck_RCL_QX200.apfpack or VeriCheck_RCL_QX600.apfpack from the File Explorer window, as appropriate for the ddPCR system being used. Click Open to import the APF.
- APFs can also be deleted from this tab by checking the checkbox next to an APF and clicking Delete Package.

See Figure 6 for a summary of steps.

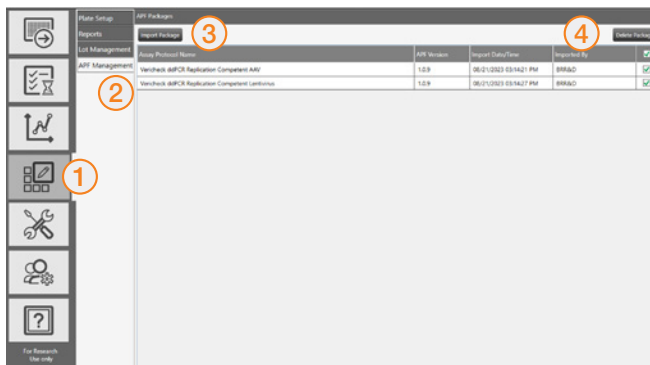


Fig. 6. Steps to import an APF package into QX Manager Software Premium Edition.

Data Acquisition Using an APF

1. Navigate to the Add Plate tab, click Add Plate, and add the 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 Lentivirus APF (Figure 7).

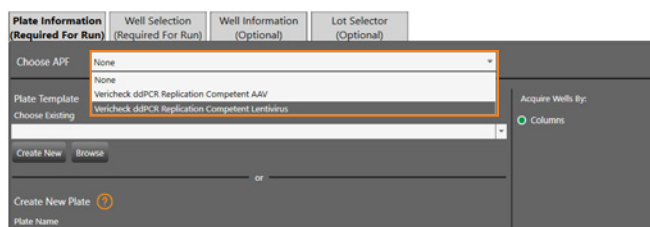


Fig. 7. Selecting an APF for use.

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 8) and designate the following:
 - Well Type: RCL
 - 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)

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.

- c. All other fields will automatically be filled in with the appropriate settings for the Vericheck ddPCR RCL Kit.
 - d. Press Apply and then Save the template.
6. Press Start Run.

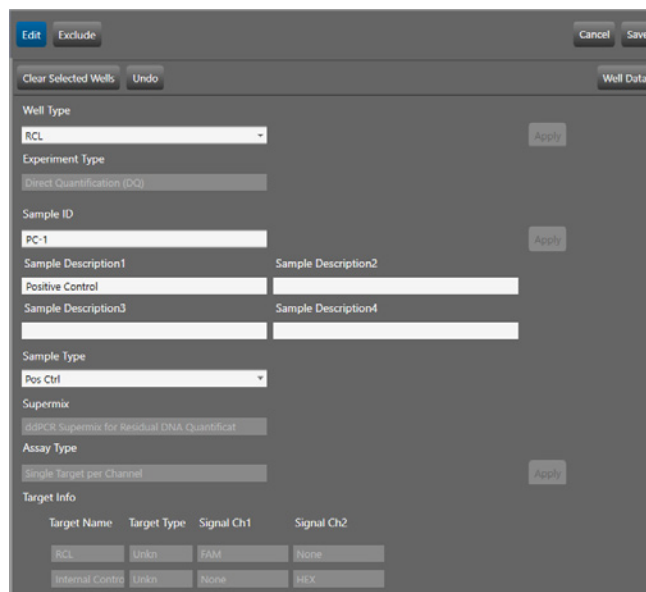


Fig. 8. Example of plate setup in the Plate Editor screen. To ensure accurate analysis of results each well must have a unique Sample ID. Positive control wells must have the sample type Pos Ctrl.

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 for that well (Figure 9).

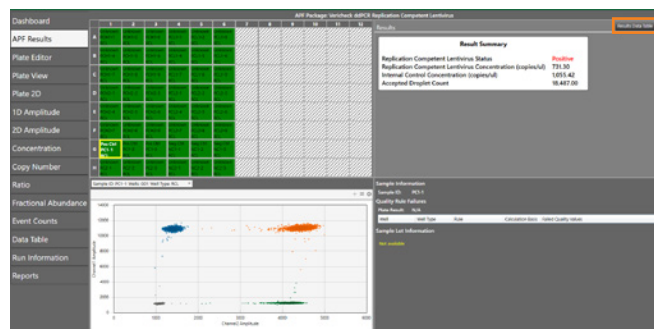


Fig. 9. APF Results tab showing results for a positive control well. Orange rectangle shows the Results Data Table button.

2. The results for all wells can be viewed together by clicking Results Data Table in the upper right corner of the screen (Figure 9, 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 10).

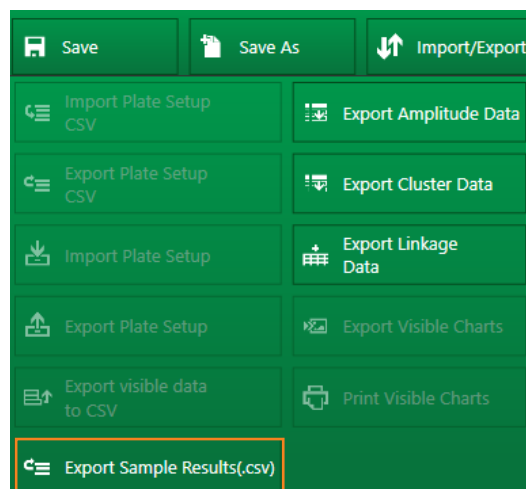


Fig. 10. Screenshot of the APF result export process. Click Export Sample Results(.csv) (orange rectangle) to export APF results.

5. To export a report, navigate to the Reports tab, ensure APF Results is checked to be included in the report (Figure 11), and click Generate Report. The Result Summary Table will be exported as part of the report.

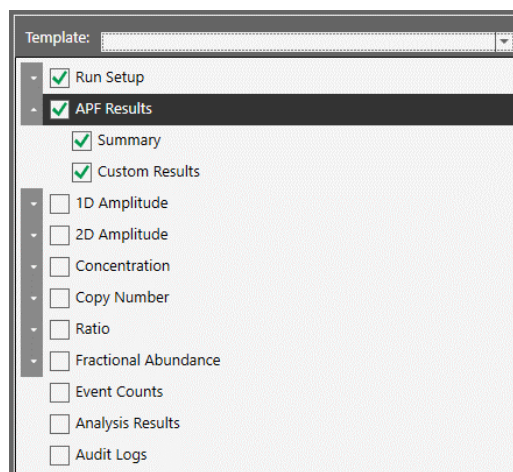


Fig. 11. On the Reports tab, ensure APF Results is checked prior to generating the report.

Calculating Percentage Recovery

If the internal control was spiked in prior to extraction, it can be used as a control for extraction efficiency. The internal control concentration for positive control, negative control, and sample wells can be viewed as part of the APF results. The following formula can be used to determine the percentage recovery of each extracted sample:

$$R = (S/C) \times 100\%$$

Where:


R = percentage recovery

S = sample well internal control concentration, copies/μl

C = average positive control and negative control well internal control concentration, copies/μl

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 based 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 RCL 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 Lentivirus APF package name and more details will open below it.

- In the Variables table, click the bolded number in the Modified column and type in the desired positivity cutoff, in copies/ μ l of the final 1x ddPCR reaction (Figure 12).
- Click Save. For all future plates, the modified value will be used as a cutoff for calling wells RCL positive.

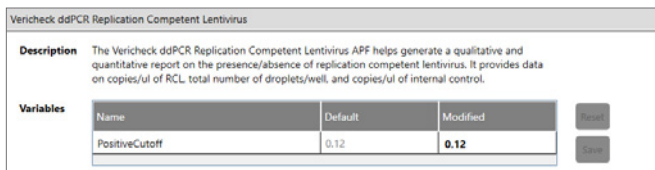


Fig. 12. VeriCheck ddPCR Replication Competent Lentivirus APF package details in the APF Management tab of QX Manager Software. The positivity cutoff for the APF can be adjusted by typing a new number into the Modified box of the Variables table.

Note: The positivity cutoff level of 0.12 copies/ μ l was determined using contrived samples that do not contain residual VSV-G contamination. Users can determine their own positivity cutoff based on their sample and experimental testing and adjust the APF custom positivity cutoff as desired.

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.

No Negative Droplets

Problem: Only high-amplitude clusters are present in sample well (Figure 13). The sample is too concentrated and is outside of the ddPCR dynamic range.

Resolution: Exclude well from analysis, dilute sample, and test again.

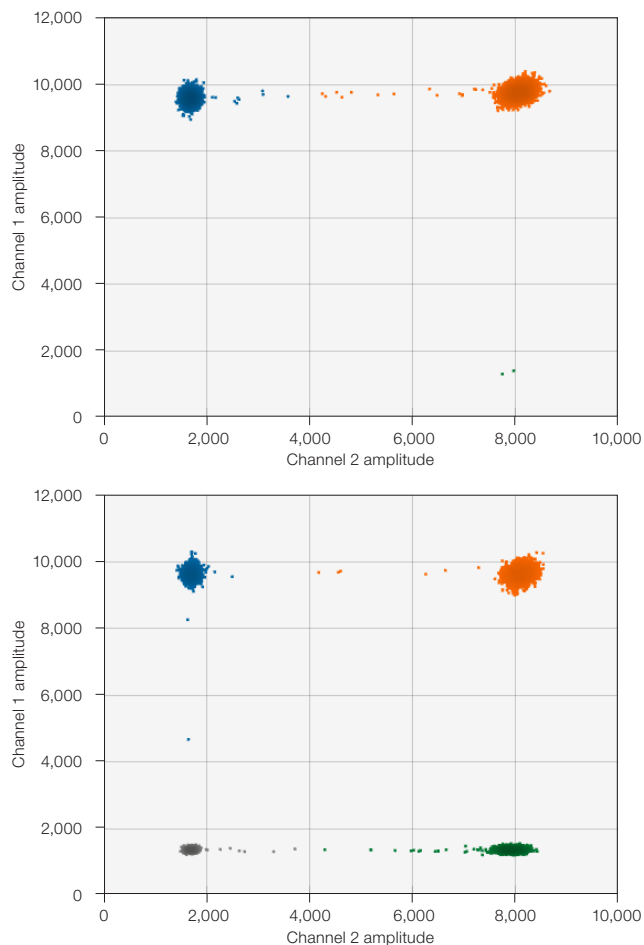


Fig. 13. No negative droplets. Problem well (top) versus normal well (bottom).

No or Low Total Droplet Counts in Sample Wells Only

Problem: Multiple replicate sample wells have a droplet count <10,000 (Figure 14). If all control wells have droplet counts ≥10,000, the issue is likely caused by inhibitors carried over from the starting matrix or extraction.

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 14). If control wells have low droplet counts, inhibition is likely not the 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.

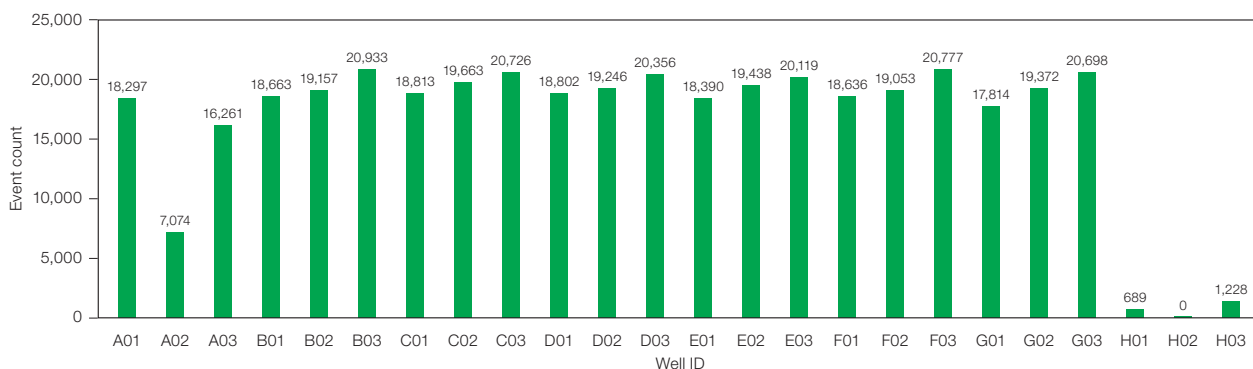


Fig. 14. Event count graph in which some wells have a total droplet count <10,000.

PCR Inhibition

Problem: Separation between clusters decreases and rain increases (Figure 15). 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.

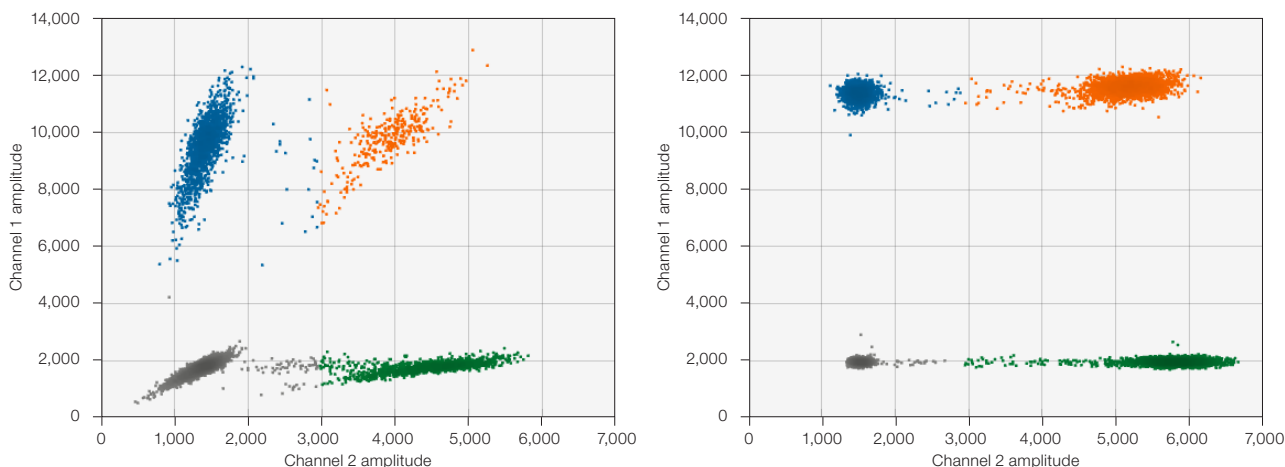


Fig. 15. PCR inhibition. Problem well (left) versus normal well (right).

Mirroring

Problem: Droplets exhibit two or more distinct sizes (Figure 16). 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.

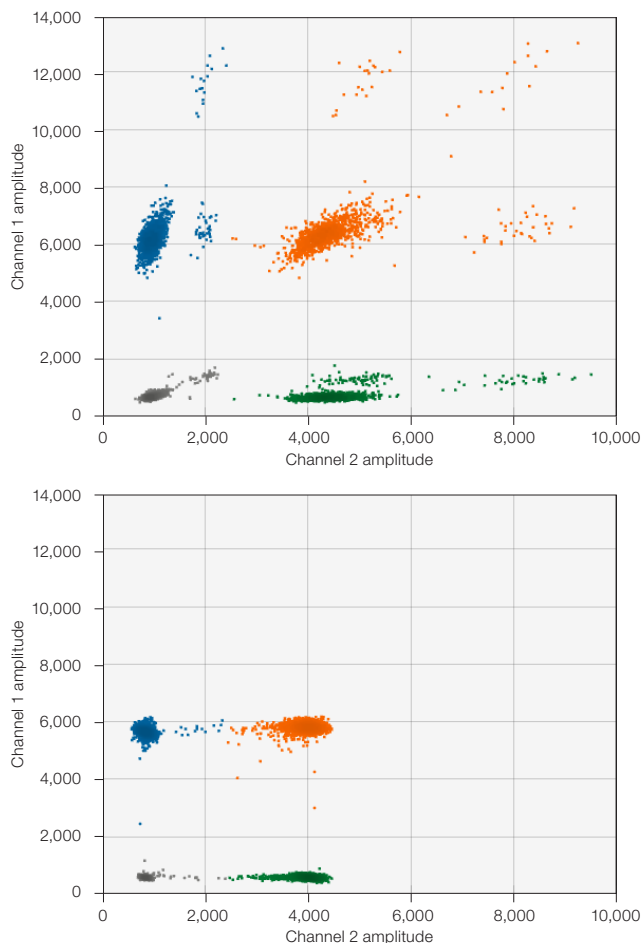


Fig. 16. Mirroring. Problem well (top) versus normal well (bottom).

Droplet Shredding

Problem: Shredded droplets appear on the diagonal through the negative droplet cluster (Figure 17).

Resolution: Exclude well from analysis or repeat Droplet Digital PCR.

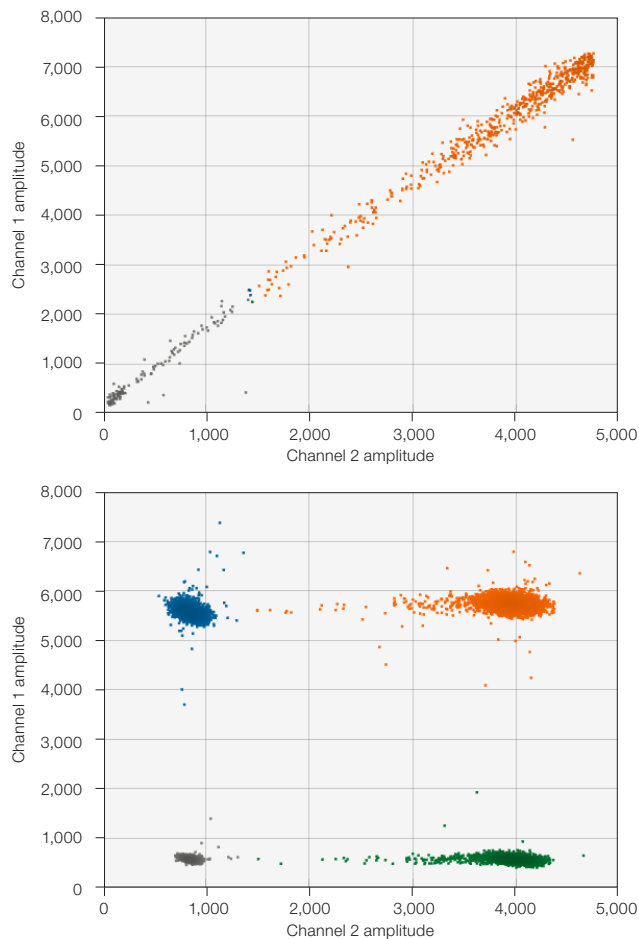


Fig. 17. Droplet shredding. Problem well (top) versus normal well (bottom).

Incorrect Autothresholding

Problem: Droplets are incorrectly thresholded using autothresholding (Figure 18).

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.

Visit [bio-rad.com/ddPCR-VeriCheck-RCL](https://www.bio-rad.com/ddPCR-VeriCheck-RCL) for more information.

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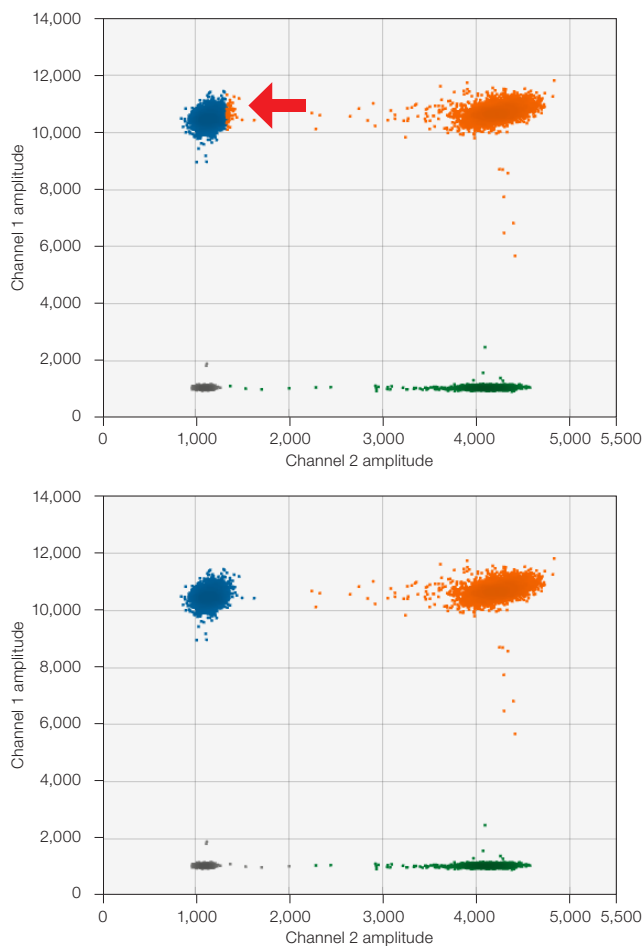


Fig. 18. Incorrect autothresholding. Incorrect thresholding indicated by arrow (top) versus correct thresholding after manual adjustment (bottom).