

VeriCheck ddPCR™ HEK293 Res DNA Quant Kit

User Guide

Catalog #12016814



BIO-RAD

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Section 1 Introduction

The VeriCheck ddPCR HEK293 Res DNA Quant Kit is designed for the quantification of residual host cell DNA (HCD) using Droplet Digital™ PCR (ddPCR), which provides quantitative results without use of extraction methods. HCD carried over during the process of manufacturing therapeutic proteins and vaccines poses safety concerns and must not exceed levels established by regulatory agencies such as the U.S. Food and Drug Administration and the World Health Organization.

Section 2 Kit Contents

This kit uses a three-well test and is sufficient for 100 reactions. Keep at –20°C for long term storage or at 4°C for up to 2 weeks. The kit's contents are listed in Table 1.

Table 1. Kit contents.

Reagent	Vials	Volume
ddPCR Supermix for Residual DNA Quantification (2x)	2	0.75 ml
ddPCR HEK293 Quantification Assay (20x)	1	150 µl
ddPCR HEK293 Internal Control (20,000 copies/µl)	1	140 µl
HEK293 Quant Positive Control	1	80 µl
Nuclease-Free H ₂ O (negative control)	1	1.5 ml

Section 3 Required Equipment, Reagents, and Consumables

Table 2 lists required materials not included in the kit.

Table 2. Additional required materials.

Instrument	Consumables and Reagents*
<ul style="list-style-type: none"> ▪ QX200 Droplet Generator (Bio-Rad™ Laboratories, Inc., catalog #1864002) or 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"> ▪ Droplet Generation Oil for Probes (#1863005) or Automated Droplet Generation Oil for Probes (#1864110) ▪ ddPCR Droplet Reader Oil (#1863004) ▪ ddPCR 96-Well Plates (#12001925) ▪ DG8 Cartridges (#1864008) or DG32 Automated Droplet Generator Cartridges (#1864108, 1864109) ▪ ddPCR Buffer Control for Probes (#1863052) ▪ Pipet Tips for AutoDG™ System (#1864120, 1864121) ▪ PCR Plate Heat Seal (#1814040)
<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)

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

Section 4

Sample Preparation: Sample Dilution and DNA Loading

Most samples follow an extraction-free workflow and do not need to be processed using a sample preparation kit. Samples containing high levels of ddPCR inhibitors or background genomic DNA may require dilution prior to setting up the reaction mix. Table 3 contains suggested dilution ranges for samples that typically occur in different phases of the production process. Examples of early-workflow samples are cell lysate and cell culture media. Examples of mid-workflow samples would be samples in purification stages. Finished products such as chimeric antigen receptor (CAR) T cells would be late-workflow samples. The dilution range of adeno-associated virus (AAV) samples is dependent on the storage buffer and concentration of AAV particles in the starting sample. To determine the optimal dilution for a particular sample, the user can start with the ranges in Table 3 and do a series of dilutions until a unique range for the sample has been determined. See Appendix B for detailed information on allowable concentrations for common inhibitors.

Table 3. Dilution recommendations.

Workflow Stage	Sample Type	Dilution
Early	Cell culture media, cell lysate	3–30x
Middle	Wash buffers used in purification	4–20x
Late	CAR T cells in PBS and HSA	3x
	AAV in PBS	3x
	AAV in water	1x

AAV, adeno-associated virus; CAR, chimeric antigen receptor; HSA, human serum albumin; PBS, phosphate buffered saline.

High levels of cells or unfragmented DNA (including non-HEK DNA) can act as an inhibitor and may require restriction enzyme digestion or dilution. Guidance on DNA loading is listed in Table 4.

Table 4. DNA loading guidance. HEK DNA refers specifically to the HEK DNA to be detected by this kit. Total DNA refers to the total amount of DNA in the sample, including normal human (non-HEK) DNA.

Sample Type	DNA Load	Equivalent Number of Cells
HEK DNA	0.8 pg to 50 ng	0.1 to 5e3 cells (triploid cells)
Undigested total DNA	Up to 66 ng	Up to 1e4 cells (diploid cells)
Digested* total DNA	Up to 1 µg digested	Up to 1.7e5 cells (diploid cells)

* Add 0.5 µl CviQ1 or HindIII restriction enzyme (at 10,000 units/ml) to each 20 µl ddPCR reaction to digest DNA within droplets. In this case, add only 7.5 µl sample to ensure a total volume of 20 µl per reaction.

Section 5

Reaction Setup

- Determine the plate layout prior to setting up the reaction mix. Each sample and control should have three replicate wells that can be merged to form a complete three-well test. An example plate configuration is shown in Figure 1.

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	Pos Ctrl	Pos Ctrl	Pos Ctrl	#7	#7	#7						
B	Sample #1	Sample #1	Sample #1	#8	#8	#8						
C	Sample #2	Sample #2	Sample #2	#9	#9	#9						
D	Sample #3	Sample #3	Sample #3	#10	#10	#10						
E	Sample #4	Sample #4	Sample #4	#11	#11	#11						
F	Sample #5	Sample #5	Sample #5	#12	#12	#12						
G	Sample #6	Sample #6	Sample #6	#13	#13	#13						
H	Neg Ctrl	Neg Ctrl	Neg Ctrl	#14	#14	#14						

Fig. 1. Example plate configuration for controls and 14 samples. Columns 7–12 can be used to run additional samples. **Three replicate wells** should be included for every sample and control.

- Sample:** DNA sample is added to the ddPCR reaction. The HEK293 signal will be read in channel 1 (FAM). Internal control is added to the master mix and will be read in channel 2 (HEX).
 - Pos Ctrl (HEK293 Quant Positive Control):** Instead of sample, positive control is added to the ddPCR reaction. The positive control signal is detected in channel 1 (FAM). Internal control is added to the master mix and will be read in channel 2 (HEX).
 - Neg Ctrl (Nuclease-Free H₂O [negative control]):** Instead of sample, nuclease-free water is added to the ddPCR reaction. Internal control is added to the master mix and will be read in channel 2 (HEX).
- 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 contents at the bottom of each tube.

Note: Thorough vortexing (maximum speed for 15 sec) of each component at this step is essential.

- Most samples follow an extraction-free workflow and do not need to be processed using a sample preparation kit. Ensure samples are thoroughly mixed. If necessary, dilute samples to the desired concentration before setting up the reaction mix. The recommended input HEK DNA is 0.8 pg–50 ng per well. See Appendix B for additional sample dilution recommendations based on common ddPCR inhibitors.
- Assemble a master mix containing supermix, quantification assay, and internal control, according to the master mix setup guidelines in Table 5. Vortex the master mix thoroughly at maximum speed for 15 sec.

Table 5. Preparation of the master mix containing supermix, quantification assay, and internal control.

Component	Volume per Reaction, μ l	Volume per Reaction, μ l	Volume per 96 Reactions + Overage*, μ l
	1 Well	1 Well + Overage*	30 Samples, 3 Wells Each**
ddPCR Supermix for Residual DNA Quantification	10	11	1,320
HEK293 Quantification Assay	1	1.1	132
HEK293 Internal Control	1	1.1	132
Total volume	12	13.2	1,584

* Prepared with overage to account for liquid loss during pipetting steps.

** Volumes in this column are for a total of 96 wells (30 samples [3 wells each], positive control [3 wells], and nuclease-free H₂O/negative control [3 wells]).

5. Prepare reaction mix for each set of three replicate wells by combining master mix (from step 4) and the sample or control.
 - a. Use the volumes given in Table 6 to prepare the negative control, positive control, and the sample reaction mixes.
 - b. Mix each tube thoroughly by vortexing at maximum speed for 15 sec, then 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 ddPCR 96-Well 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.

Table 6. Preparation of reaction mix.

Component	Volume per Reaction, μ l	Volume per Reaction, μ l	Volume for 3 Wells + Overage, μ l
	1 Well	1 Well + Overage*	3 Wells + Overage*, μ l
Master mix	12	13.2	48
Sample/positive control/nuclease-free H ₂ O (negative control)	8	8.8	32
Total volume	20	22	80

* Prepared with overage to account for liquid loss during pipetting steps.

6. Transfer the reaction mix or buffer control to the appropriate droplet generation cartridge and generate droplets based on the system in use.
 - a. **For the QX200 Droplet Digital PCR System:**
 - i. Load 20 μ l 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.
 - ii. Add 20 μ l of buffer control to any unused wells in a column from which droplets will be generated.
 - iii. Load 70 μ l of Droplet Generation Oil for Probes into the oil wells. For detailed instructions, refer to the QX200 Droplet Generator Instruction Manual ([10031907](#)).
 - b. **For the QX200 AutoDG Droplet Digital PCR System:**
 - i. Load 22 μ l of each reaction mix into the wells of a ddPCR 96-Well Plate.
 - ii. Add 22 μ l of 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.
 - iii. 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.
 - iv. Place the sealed plate in the Automated Droplet Generator and follow instructions in the Automated Droplet Generator Instruction Manual ([10043138](#)).
 - c. **For the QX ONE ddPCR System:**
 - i. Load 20 μ l of each reaction mix into the wells of a GCR96 Cartridge.
 - ii. Droplet generation occurs in sets of two columns (for example, droplets for columns 1 and 2 are generated simultaneously). Add 20 μ l of buffer control to any unused wells in each set of columns from which droplets will be generated.

- iii. Seal the plate using the PX1 PCR Plate Sealer at 180°C for **0.5 sec**, flip the plate 180° and repeat sealing, allow to cool briefly, and centrifuge at 1,150 rcf for **30 sec**.
- iv. Follow subsequent instructions for loading the plate, as specified in the QX ONE Droplet Digital PCR System and QX ONE Software User Guide (10000116512). Use the appropriate thermal cycling conditions specified in Table 7.

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:** Residual DNA Quantification (RDQ)
- **Sample Descriptions:** Determined by user
- **Sample Type:** Determined by user
- **Conversion Factor:** 2.75 (HEK293; HEK293T)
 - See Appendix C for more information
- **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

Click **Apply** and then **Save** to save the template. Click **Start Run**. See Figure 2 for an example.

Section 6

Thermal Cycling

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

- **For the QX200 ddPCR System**, after 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. 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 specified in Table 7
- **For the QX200 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 specified in Table 7
- **For the QX ONE ddPCR System**, thermal cycling is integrated into and sequentially performed by the system itself. Hence, no additional equipment or sample handling is required for this step

Table 7. 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 ddPCR System (optional)	4	∞	1	
	QX ONE ddPCR System (required)	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.

Section 7

Data Acquisition

Follow the instructions for data acquisition based on the system in use.

- **For the QX200 ddPCR System and the QX200 AutoDG ddPCR System using QX Manager Software Standard or Regulatory Edition version 1.2 or higher**, place the sealed 96-well plate in the QX200 Droplet Reader and refer to the QX200 Droplet Reader and QX Manager Software Regulatory Edition User Guide ([10000107224](#)) or the QX200 Droplet Reader and QX Manager Software Standard Edition User Guide ([10000107223](#)) for plate setup. See Figure 2 for an example.

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:** Residual DNA Quantification (RDQ)
- **Sample Descriptions:** Determined by user
- **Sample Type:** Determined by user
- **Conversion Factor:** 2.75 (HEK293, HEK293T)
- See Appendix C for more information

- **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

Click **Apply** and then **Save** to save the template. Click **Start Run**. See Figure 2 for an example.

- **For the QX ONE ddPCR System using QX ONE Software version 1.1 or higher**, data acquisition is integrated into and sequentially performed by the system itself. Hence, no additional equipment or sample handling is required for this step.

The screenshot displays the Plate Editor interface with the following configuration:

- Experiment Type:** Residual DNA Quantification (RDQ)
- Sample Description1:** Pos Ctrl
- Sample Description2:** (empty)
- Sample Description3:** (empty)
- Sample Description4:** (empty)
- Sample Type:** Pos Ctrl (highlighted with a yellow rectangle)
- Conversion Factor:** 2.750
- Supermix:** ddPCR Supermix for Residual DNA Quantificat
- Assay Type:** Single Target per Channel
- Target Info Table:**

	Target Name	Target Type	Signal Ch1	Signal Ch2
–	Pos Ctrl	Unkn	FAM	None
–	Internal Ctrl	Unkn	None	HEX
+				
- Well Notes:** (empty text area)
- Plate Notes:** (empty text area)

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

Section 8

Data Analysis and Result Interpretation

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**. It then applies those thresholds to all currently selected wells. Refer to the QX200 Droplet Reader and QX Manager Software Regulatory Edition User Guide (10000107224) or the QX200 Droplet Reader and QX Manager Software Standard Edition User Guide (10000107223) for detailed instructions about data analysis. QX ONE Software 1.1 is not compatible with positive control–based autothresholding. See Positive Control–Based Manual Thresholding section below.

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

Important: At least one of the selected wells must have sample type **Pos Ctrl** in order to access 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. Mark as **Pos Ctrl** the positive control wells from only one assay at a time. Positive control wells from all other assays on the plate should be marked as sample type **Unknown**. Use the Sample Type dropdown menu and click **Apply** to mark selected wells.

After thresholding all wells in the first assay, return to the Plate Editor tab and change the positive control wells for the first assay to Sample Type **Unknown**. Then repeat the thresholding tips above for the next assay. Repeat this process until all assays are thresholded.

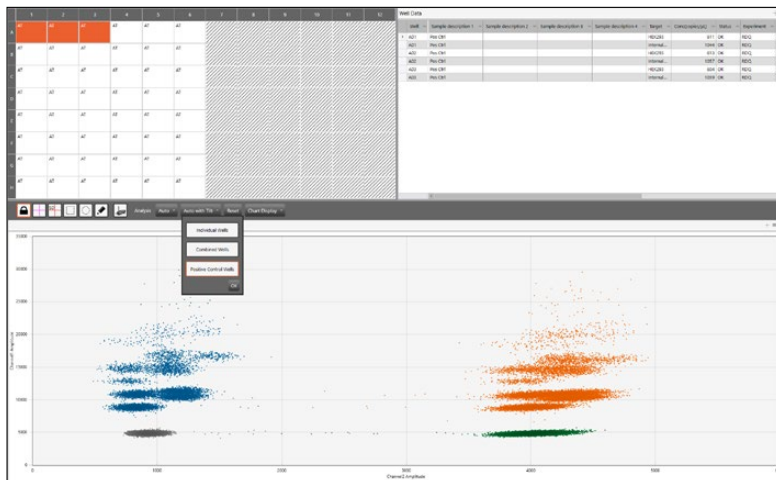


Fig. 3. 2D Amplitude screen when using positive control–based autothresholding with tilt.

Positive Control–Based Manual Thresholding

QX ONE Software version 1.1 does not support autothresholding. 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. Click the **Auto with Tilt** button and select **Combined Wells** at the bottom of the dropdown menu. This will tilt-correct all selected wells.
2. Select the positive and negative control wells for one assay. Manually threshold the control wells using the pink manual thresholding tool on the 2D Amplitude tab.
3. Copy the manual threshold applied to the control wells and paste it into all other wells with the same assay (the sample wells).
4. If multiple assays are run on the same plate, repeat steps 1–3 for each assay.

Tip: Refer to the QX ONE Software Regulatory Edition User Guide ([10000116656](#)) and the Droplet Digital PCR Applications Guide ([bulletin 6407](#)) for detailed instructions and best practices for setting manual thresholds.

Quality Check

1. Navigate to the Event Counts tab.

Recommended best practice: If the 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. Do not use poor-quality positive control well(s) as thresholding controls. See Figures 4 and 5 for example positive and negative control well 2D plots.

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

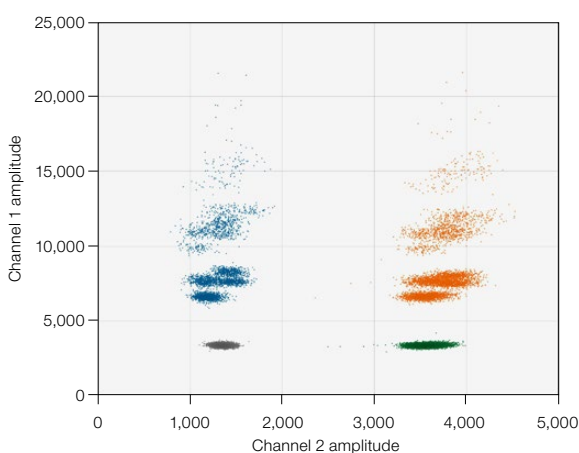


Fig. 4. Example positive control well.

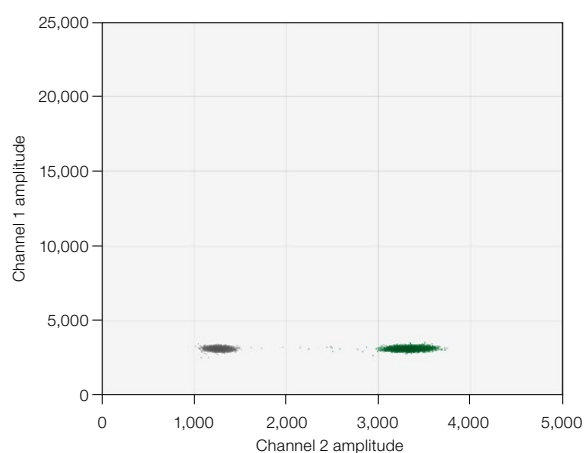


Fig. 5. Example negative control well.

3. Examine the replicate sample wells for uniformity and examine any outliers for problems. Do not include problem wells in the final analysis. If any clusters are cut off by the thresholds (for example, see Figure 16), the thresholds should be manually adjusted. See the Troubleshooting section for more details.
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 FAM (channel 1) concentration of 344–1,375 copies/μl (cp/μl) and a HEX (channel 2) concentration of 800–1,200 cp/μl. Negative control wells are expected to have a FAM concentration of 0 cp/μl and a HEX concentration of 800–1,200 cp/μl. Sample wells are expected to have a HEX concentration of 800–1,200 cp/μl. When digested with CviQI, HEX concentration will double if digestion was successful.
5. If all replicate wells have similar concentrations, merge the wells for the final analysis. Refer to the QX200 Droplet Reader and QX Manager Software Regulatory Edition User Guide (10000107224) or QX200 Droplet Reader and QX Manager Software Standard Edition User Guide (10000107223) or the QX ONE Software Regulatory Edition User Guide (10000116656) for a detailed explanation of merged wells.

Result Interpretation

1. To export data, navigate to the Data Table tab, click the Table Menu icon on the far right, and click **Export to CSV** from the dropdown menu (Figure 6).
2. The concentration of residual HEK293 DNA is given in the Molecular Weight(pg/μL) column.
3. Calculate DNA concentration of original sample using the equation:

$$U = M \times 20/S \times DF$$

where

U = pg/μl of original sample

M = Molecular Weight(pg/μL) reported in software

S = μl of sample added per 20 μl well

DF = dilution factor (if original sample was diluted)

Well	Molecular Weight(pg/μL)	Status	Status Reason	Experiment	SampleType	TargetType	Supermix	DyeName(s)	Copies/20μlWell	TotalConfMax	TotalConfMin	PoissonConfMax
M01	48			RDQ	PosCtrl	Unknown	ddPCR Sup...	FAM		140	124	
M01	47.3			RDQ	PosCtrl	Unknown	ddPCR Sup...	HEX		133	127	
M02	0			RDQ	Unknown	Unknown	ddPCR Sup...	FAM	0.0543	0	0	0
M02	0			RDQ	Unknown	Unknown	ddPCR Sup...	HEX	0.0543	0	0.0543	0
M03	48.9			RDQ	Unknown	Unknown	ddPCR Sup...	FAM		138	131	138
M03	47.3			RDQ	Unknown	Unknown	ddPCR Sup...	HEX		133	127	133
M04	0			RDQ	Unknown	Unknown	ddPCR Sup...	FAM	0.0569	0	0.0569	0
M04	0			RDQ	Unknown	Unknown	ddPCR Sup...	HEX	0.0569	0	0.0569	0
M05	46.7			RDQ	Unknown	Unknown	ddPCR Sup...	FAM		135	123	132
M05	45.7			RDQ	Unknown	Unknown	ddPCR Sup...	HEX		140	114	129
M06	0			RDQ	Unknown	Unknown	ddPCR Sup...	FAM	0.0563	0	0.0563	0
M06	0			RDQ	Unknown	Unknown	ddPCR Sup...	HEX	0.0563	0	0.0563	0
M07	51.8			RDQ	Unknown	Unknown	ddPCR Sup...	FAM		150	135	146
M07	50.3			RDQ	Unknown	Unknown	ddPCR Sup...	HEX		147	130	142
M08	0			RDQ	Unknown	Unknown	ddPCR Sup...	FAM	0.0572	0	0.0572	0
M08	0			RDQ	Unknown	Unknown	ddPCR Sup...	HEX	0.0572	0	0.0572	0
M09	45.4			RDQ	Unknown	Unknown	ddPCR Sup...	FAM		132	117	128
M09	44.6			RDQ	Unknown	Unknown	ddPCR Sup...	HEX		126	120	126
M10	0			RDQ	Unknown	Unknown	ddPCR Sup...	FAM	0.0576	0	0.0576	0
M10	0			RDQ	Unknown	Unknown	ddPCR Sup...	HEX	0.0576	0	0.0576	0
M11	48.3			RDQ	Unknown	Unknown	ddPCR Sup...	FAM		137	128	136
M11	47.2			RDQ	Unknown	Unknown	ddPCR Sup...	HEX		133	127	133

Fig. 6. Screenshot of data export example.

Section 9 Appendices

Appendix A. Performance Specifications

Limit of Blank/Limit of Detection/Limit of Quantification

The kit was tested with nuclease-free H₂O (negative control). Zero positive droplets were observed in ≥95% of tests, supporting a limit of blank (LOB) of zero. A low-concentration sample was then serially diluted in 1:1 steps. The limit of detection (LOD) was set to the lowest concentration sample detected above the LOB in ≥95% of tests; the limit of quantification (LOQ) was set to the lowest concentration detected above the LOB in ≥99% of tests. The sample at approximately 0.8 pg/well was detected in >95% of tests, making it the LOD. The sample at approximately 8.0 pg/well was detected in >99% of tests, making it the LOQ. These data are summarized in Table 8.

Table 8. LOB/LOD/LOQ data summary.

Input, pg/well	Original Sample, pg/μl	n	n Detected Above LOB	Detection Rate, %	Mean Measured Copies per Test
8.0	1.0	48	48	100	61.8
2.4	0.3	48	47	98	16.5
1.6	0.2	48	47	98	11.3
0.8	0.1	48	47	98	5.8
0.24	0.03	48	35	73	1.8

Specificity

The kit does not detect DNA from *E. coli*, Chinese hamster ovary (CHO), normal human, or Vero cells with >99.99% specificity.

Reproducibility

Six samples were tested across multiple instruments, operators, days, and kit lots. The coefficient of variation (CV) for all tests fell below 10% for all samples. Reproducibility data are summarized in Table 9.

Table 9. Reproducibility data summary.

Sample	n	Mean Concentration, cp/μl	CV, %
HEK293 Quant Positive Control — high	24	696.5	7.6
HEK293 Quant Positive Control — medium	24	70.8	4.0
HEK293 Quant Positive Control — low	24	6.95	6.5
HEK293 Quant Positive Control with PBS and HSA	24	70.2	6.0
AAV	24	651.9	4.3
Sonicated DNA	24	1,348.6	4.5

AAV, adeno-associated virus; CV, coefficient of variation; HSA, human serum albumin; PBS, phosphate buffered saline.

Dynamic Range

The kit is linear throughout the dynamic range from ~1 pg/well to 50 ng/well, or 1.0–6,875 copies/μl (Figure 7).

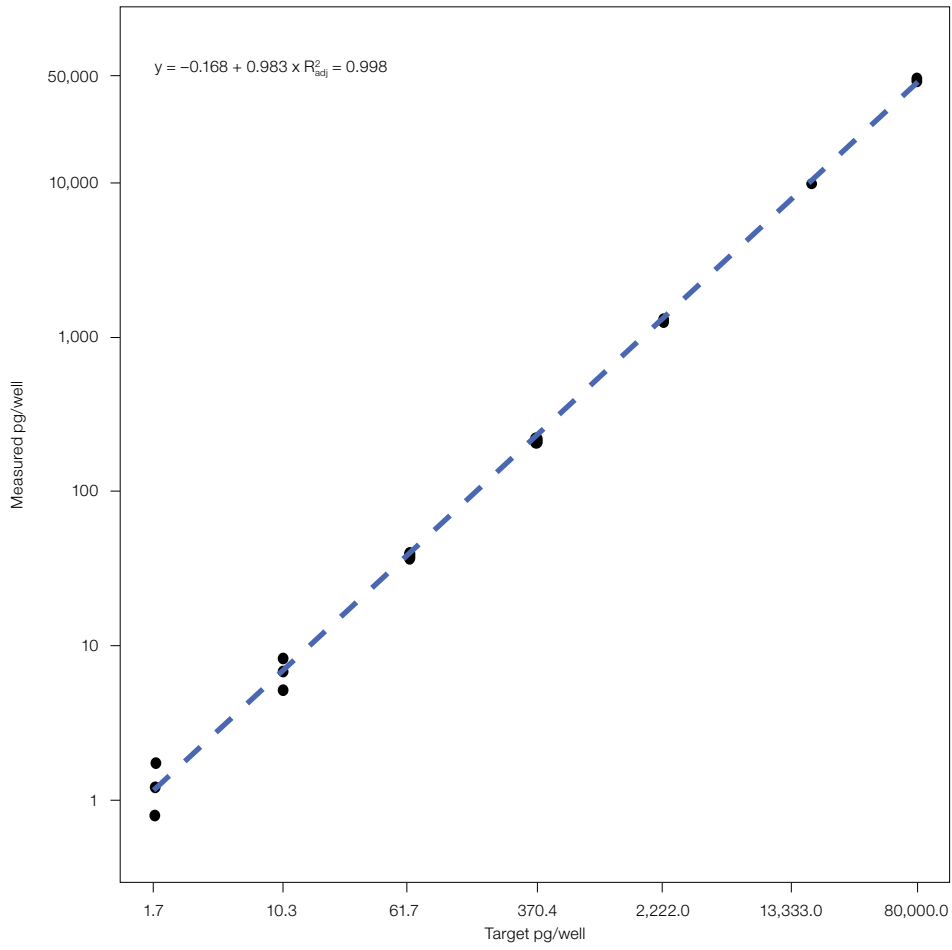


Fig. 7. Linearity.

Appendix B. Inhibitors and Extraction-Free Workflow

Samples containing ddPCR inhibitors may require dilution to meet the concentrations listed in Tables 10–12. Using samples with greater than the allowable concentration will impact results. Given an input of 8 μ l per 20 μ l reaction, the concentration in the sample is 2.5x the concentrations listed in Tables 10–12. Water is recommended for dilution, but other buffers containing low levels of Tris-EDTA, Poly(A) (Sigma-Aldrich Corporation), or Pluronic F-68 Non-Ionic Surfactant (Thermo Fisher Scientific Inc.) are also compatible. Samples containing multiple sources of inhibitors may require further dilution. Inhibitors were tested individually and not in combination with other inhibition agents. The concentrations listed in Tables 10–12 are for extraction-free samples.

Table 10. Chemical inhibitors.

Inhibitor	Maximum Allowable Concentration in Well
SDS	0.01%
Tween 20	0.01%
NP-40	0.01%
Pluronic F-68	0.625%
PEI	108 μ g/ μ l
Triton X-100	0.01%
EDTA	1.25 mM
DMSO	2.5%

DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; PEI, polyethyleneimine; SDS, sodium dodecyl sulfate.

Table 11. Workflow/in-process inhibitors.

Inhibitor	Maximum Allowable Concentration in Well
EMEM Complete Media	20%
NaCl*	26 mM
Lysate (0.2% SDS)	1.25%
Histidine, sucrose, Tween 20	5.6 mM, 93.1 mM, 0.01%

* NaCl was formulated with a Tris buffer and not tested as an individual component.

Table 12. Sample inhibitors.

Inhibitor	Maximum Allowable Concentration in Well
AAV	>1.25e12 GC/ml
PBS + 2.5% HSA	15%

AAV, adeno-associated virus; GC, gene copies; HSA, human serum albumin; PBS, phosphate buffered saline.

Appendix C. Understanding Conversion Factor

The conversion factor (CF) provides a link between observed copies of DNA and the total mass of DNA. The conversion factor is calculated by comparing the copy number of the HEK293-specific region with a stable genomic reference. For HEK293A and HEK293T, this should be set to 2.75 cp/pg. HEK293 is a pseudotriploid cell line with a total genome mass of approximately 9.6 pg. For a stable target on a trisomic chromosome,

$$CF = \frac{3 \text{ copies}}{\text{genome}} \times \frac{\text{genome}}{9.6 \text{ pg}} = 0.31$$

To calculate the conversion factor for another HEK293 line, test an identical sample with the VeriCheck HEK293 Res DNA Quant Kit, an *RPP30* reference assay (dHsaCP2500350), an *IRAK4* reference assay (dHsaCP2506307), and an *MRPS5* reference assay (dHsaCP2506282), with nine wells of each assay and approximately 10 ng of DNA per well. Samples should have only clean HEK293 DNA; background human DNA will interfere with measurement.

All assays should be prepared and thermal cycled according to the package insert and digested in droplets by adding 0.5 μ l of CviQI restriction enzyme directly to the master mix.

Calculate the average concentration for each assay and the CF using the equation below. If one of *RPP30*, *IRAK4*, or *MRP25* is 30% lower or 25% higher than the other two, that assay should be discarded, as it indicates karyotypic drift. In this case, calculate the average of the remaining two assays.

$$CF = \frac{3 \times 0.31 \times \text{concentration}_{\text{HEKQuant}}}{(\text{concentration}_{\text{RPP30}} + \text{concentration}_{\text{IRAK4}} + \text{concentration}_{\text{MRPS5}})}$$

Section 10

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 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 (Figure 8).

Conc(copies/μL)	Molecular Weight(pg/μL)	Status	Status Reason	Experiment
No Call		CHECK	Droplet Maximum Amplitude Exceeded	RDQ
No Call		CHECK	Droplet Maximum Amplitude Exceeded	RDQ
No Call		CHECK	Droplet Maximum Amplitude Exceeded	RDQ
No Call		CHECK	Droplet Maximum Amplitude Exceeded	RDQ
No Call		CHECK	Droplet Maximum Amplitude Exceeded	RDQ
No Call		CHECK	Droplet Maximum Amplitude Exceeded	RDQ
0		OK	Reason for CHECK status: Droplet Maximum Amplitude Exceeded To View Concentration: Review Your Data Use Manual Analysis Tools	
981		OK		
0		OK		
969		OK		RDQ
0		OK		RDQ
972		OK		RDQ

Fig. 8. Wells with Droplet Maximum Amplitude Exceeded error will have No Call as the concentration (cyan oval) and CHECK as the status.

Solution: Select a positive control well, copy the autothreshold applied to the control well, and paste it onto all wells with this issue. The software will now display a concentration for those wells and have the status **Manual** (Figure 9).

Conc(copies/μL)	Molecular Weight(pg/μL)	Status	Status Reason	Experiment
679		Manual	Droplets Analyzed Manually	RDQ
971		Manual	Droplets Analyzed Manually	RDQ
693		Manual	Droplets Analyzed Manually	RDQ
956		Manual	Droplets Analyzed Manually	RDQ
677		Manual	Droplets Analyzed Manually	RDQ
933		Manual	Droplets Analyzed Manually	RDQ
0		OK		RDQ
969		OK		RDQ
0		OK		RDQ
989		OK		RDQ
0		OK		RDQ
980		OK		RDQ

Fig. 9. Data table after the Droplet Maximum Amplitude Exceeded error has been resolved.

Tips to avoid error: Digesting samples that may contain full-length DNA or >2,000 bp fragments of DNA with CviQI restriction enzyme will reduce the likelihood that this error occurs. Add 0.5 µl CviQI or HindIII restriction enzyme (at 10,000 units/ml) to each 20 µl ddPCR reaction to digest DNA within droplets. In this case, add only 7.5 µl sample to ensure a total volume of 20 µl per reaction.

Too Much Unfragmented DNA

Problem: No distinct clusters in the 2D plot and droplet counts <10,000 (Figure 10). Wells have a high background of unfragmented DNA.

Resolution: Add 0.5 µl CviQI or HindIII restriction enzyme (10,000 units/ml) to each 20 µl ddPCR reaction to digest DNA within droplets. In this case, add only 7.5 µl sample to ensure a total volume of 20 µl per reaction.

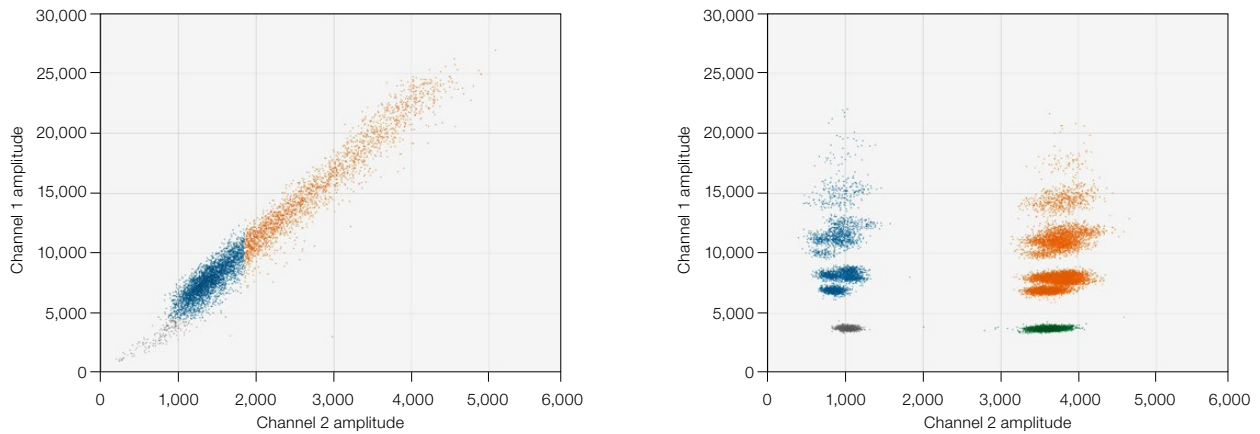


Fig. 10. 2D plots for wells containing 1 µg normal human DNA and 10 ng HEK293 DNA. **Left**, problem well where DNA is not digested versus **right**, normal well where restriction enzyme was added to master mix to digest background DNA.

No Negative Droplets

Problem: Only high-amplitude clusters are present in sample well. The sample is too concentrated (Figure 11) and is outside of the ddPCR dynamic range.

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

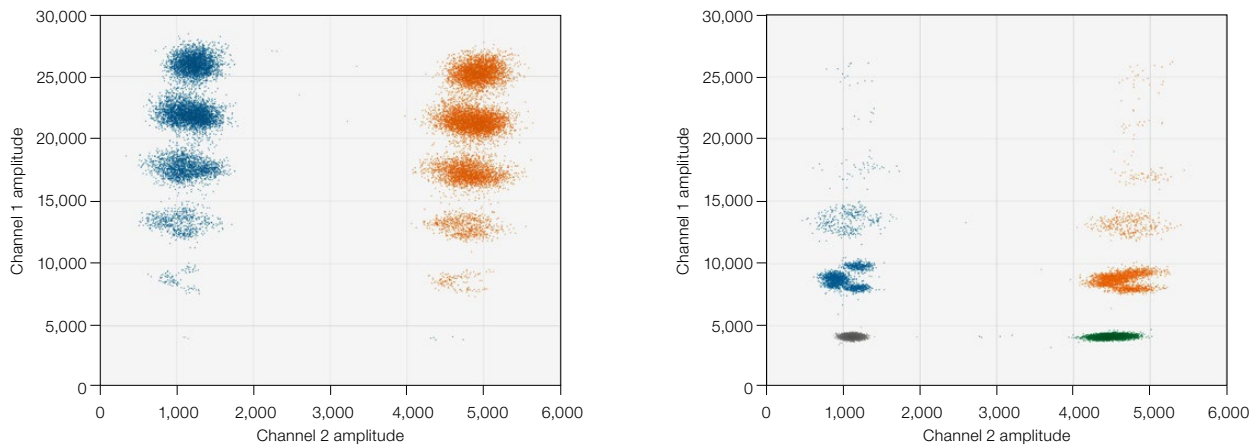


Fig. 11. No negative droplets. **Left**, problem well versus **right**, normal well.

No or Low Total Droplet Counts in Sample Wells Only

Problem: Multiple replicate sample wells have a droplet count <10,000 (Figure 12). If 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, extract the sample to eliminate inhibitors.

No or Low Total Droplet Counts in Both Sample and Control Wells

Problem: Sample and control wells have droplet counts <10,000 (Figure 12). 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. Repeat Droplet Digital PCR and wait 30 minutes or more at 4°C after thermal cycling completes to increase droplet numbers. If still a problem, call Technical Support.

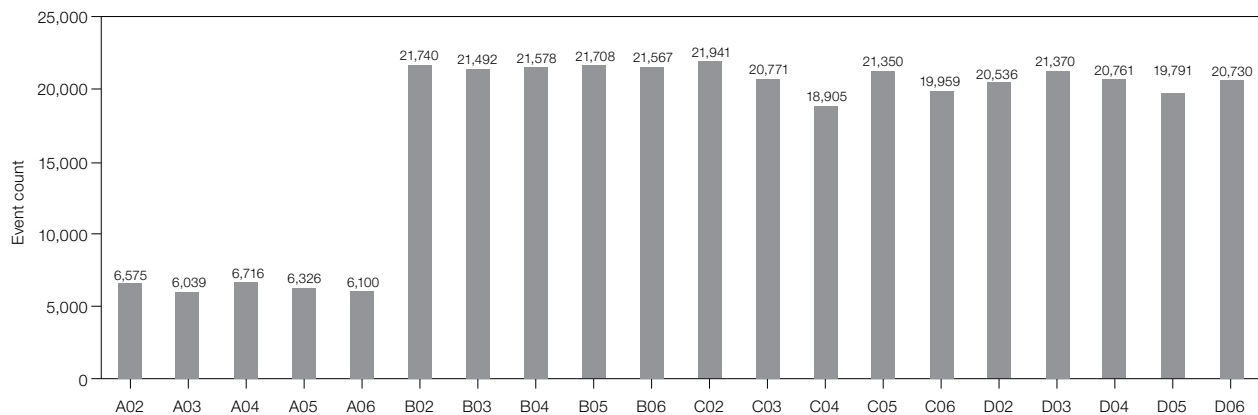


Fig. 12. Event count graph in which wells have a total droplet count <10,000.

PCR Inhibition

Problem: Separation between clusters decreases and rain increases (Figure 13).

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

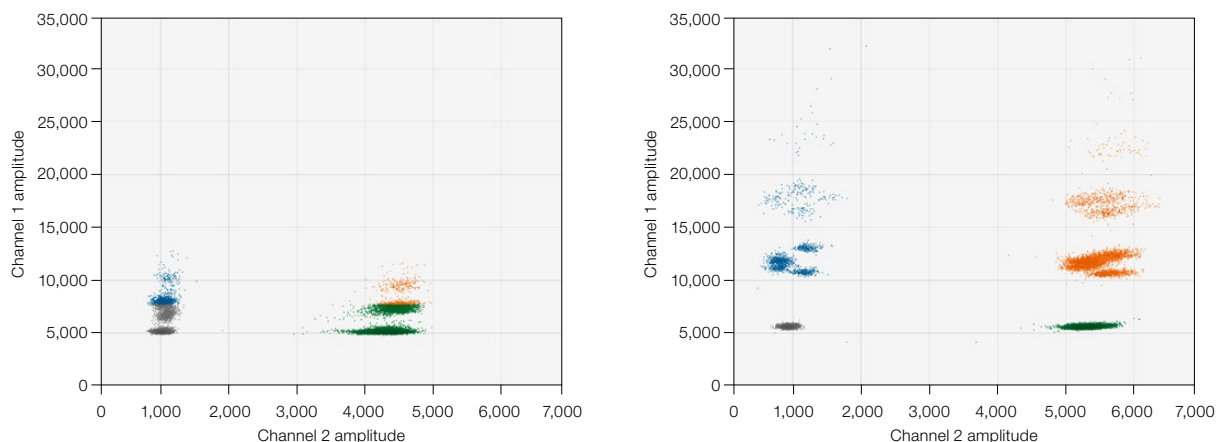


Fig. 13. PCR inhibition. Left, problem well versus right, normal well.

Mirroring

Problem: Droplets exhibit two distinct sizes (Figure 14). 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.

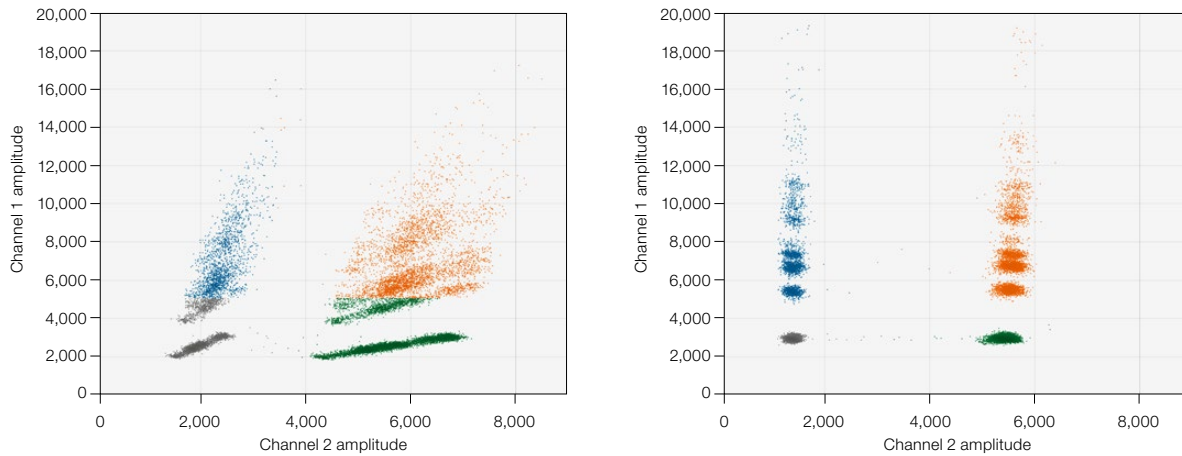


Fig. 14. Mirroring. Left, problem well versus right, normal well.

Droplet Shredding

Problem: Shredded droplets appear on the diagonal through the negative droplet cluster (Figure 15).

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

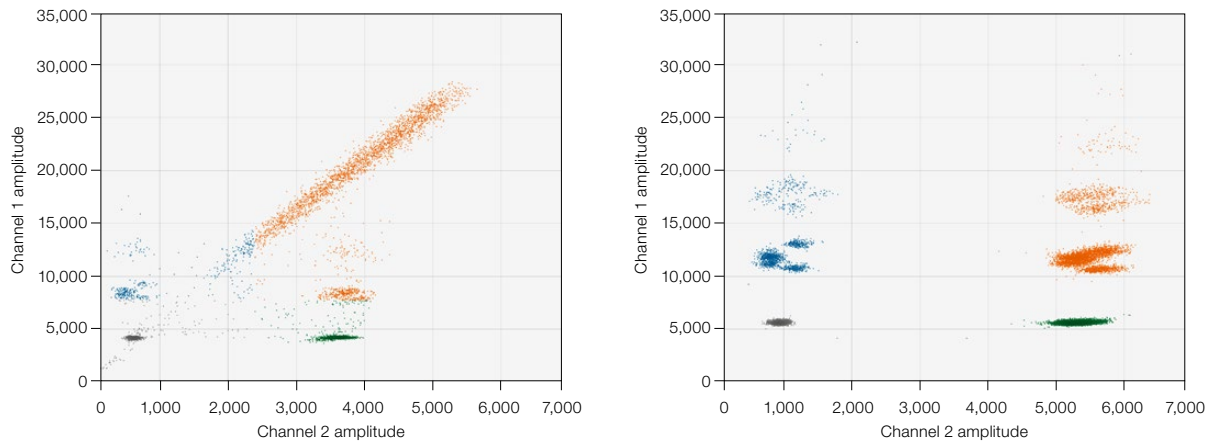


Fig. 15. Droplet shredding. Left, problem well versus right, normal well.

Incorrect Autothresholding

Problem: Droplets are incorrectly thresholded using autothresholding (Figure 16).

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 Droplet Digital PCR Applications Guide ([bulletin 6407](#)) for best practices setting manual thresholds.

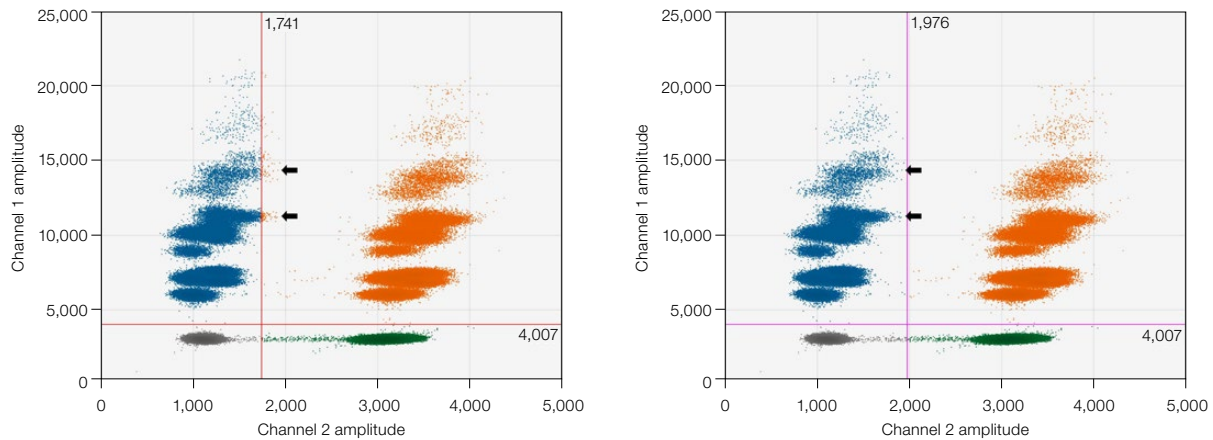


Fig. 16. Incorrect autothresholding. **Left**, incorrect thresholding indicated by arrows versus **right**, correct thresholding using positive control–based autothresholding with tilt.

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

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